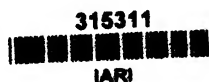




AGRICULTURAL RESEARCH INSTITUTE
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JOURNAL
OF
BACTERIOLOGY



VOLUME IV

BALTIMORE, MD.
1919

tions were planted by platinum loop from tube to tube into faintly alkaline glucose pork broth which had been previously heated to expel the oxygen.

The inoculated tubes were then placed in a partial vacuum, the surface of the medium covered with neutral paraffin oil, or placed in a hydrogen atmosphere and allowed to incubate for five to ten days at a temperature of 22 to 24°C. The nature of the growth could be observed and recorded each day without disturbing the anaerobic conditions. Cultures showing mild gas production, followed by the development of a rancid odor, were stained and examined in cover glass films. A majority of the cultures after staining were not identified, but in some tubes organisms were encountered which morphologically resembled *B. botulinus*., 0.5 mil of the growth in these tubes was administered to guinea-pigs per os. Cultures proving fatal to guinea-pigs were again heated to eliminate non-sporeforming and vegetative bacteria, and dilutions were planted in neutral glucose gelatin plates. The plates were solidified at 15°C. and glucose gelatin or agar containing glycerine 50% was poured over the surface to insure anaerobic conditions. The plates were then incubated in a partial vacuum at a temperature of 18 to 22°C. Deep colonies developing in the plates were transferred to faintly alkaline pork broth where the morphology and cultural characters were observed.

Numerous non-pathogenic, spore-forming, anaerobic microorganisms were encountered in the silage samples, no. 1 from the surface and no. 2 from the subsurface. A microorganism was isolated from the subsurface sample showing the following characteristics:

Morphology and staining properties. Straight rod-shaped organism, slightly rounded at ends, measuring 0.8 to 1.2 microns by 3 to 6 microns. Slightly motile. Occurring singly and in pairs, rarely longer chains. Intracellular spores located near one end, slightly distending or bulging the wall. Staining evenly with the ordinary anilin dyes. Positive by Gram's method.

Cultural characteristics. Strictly anaerobic, but developing under aerobic conditions in association with *Fusarium* sp. Very

sensitive to light. Optimum temperature for growth from 22° to 25°C. Growing best in media made from meat (pork), but also in decoctions made from forage plants, i.e., corn ensilage, alfalfa, oats and corn, of a faintly alkaline reaction. In media of an acid (+2.5) reaction in association with *Fusarium* sp. favorable growth took place aerobically. The addition of glucose to the medium apparently favored growth, as indicated by energetic gas production and the toxicity of the cultures. Gelatin was liquefied, with an odor of butyric acid in old cultures. Most characteristic development in plates of glucose pork gelatin of a neutral or faintly alkaline reaction. Small, round, yellowish-brown, deep colonies appeared in five to eight days. The colonies under low magnification presented a granular appearance, giving the edge a finely fringed periphery. About the margin of older colonies the area of liquefied gelatin presented numerous fine transparent rays. In the liquefied gelatin the colonies became more or less disintegrated into small irregular granular clumps. Gas was formed, splitting the medium or collecting on the surface of the liquefied areas. The odor of butyric acid was pronounced in old cultures.

The organism produced an extracellular toxin in glucose pork broth and plain pork broth of a faintly alkaline reaction. The filtered broth culture, demonstrated to be sterile by cultural methods, proved fatal to guinea-pigs per os, and following a period of incubation engendered symptoms and death in a mule subsequently to ingestion. Similar symptoms were also recognized in the experimental horses on the McLean farm from ingesting the ensilage and some of the symptoms closely resembled those presented by horses and mules suffering from *B. botulinus* intoxication. It is probable that the soluble toxin is generated even under aerobic conditions in media of an acid reaction in association with *Fusarium* sp. and other saprophytic bacteria since mixed cultures of this character proved fatal to guinea-pigs per os. Filtration experiments were not conducted to demonstrate the presence of toxin in aerobic cultures containing mixed growth. •

Pathogenesis. Guinea-pigs proved highly susceptible to broth cultures of the bacillus (ss) isolated from the McLean ensilage. 0.02 to 0.03 mil broth culture per os proved fatal in twenty-four to eighty-four hours to guinea-pigs weighing from 250 to 500 grams each. Depression, roughened coat, slow movement, tense abdominal muscles, ptialism, loss of appetite, incoördination, muscular weakness, coma, terminating in death, were observed in artificially intoxicated pigs. Two mils filtered broth culture, proved sterile by cultural methods, administered per os resulted in the death of a mule in nine days. The filtrate was ingested, disguised in wholesome feed, and was followed by manifest symptoms of depression on the seventh day. Following a period of incubation, a sleepy, dull, listless attitude developed rapidly, and partly masticated feed with excessive amounts of saliva dropped from the mouth. Paresis of the pharynx became noticeable, accompanied by profuse ptialism. Muscular weakness and incoördinate movement culminated in decumbency on the eighth day. Complete loss of appetite was noted. Respirations were increased, following intermittent periods of violent struggling. On the ninth day death occurred in coma. The clinical symptoms observed in the mule strikingly resembled symptoms displayed by animals naturally afflicted from ingesting the silage. The gross anatomic alterations observed upon autopsy were likewise suggestive and resembled changes noted in animals affected with forage poisoning in natural outbreaks of the disease.

IMMUNOLOGICAL AND SEROLOGICAL RELATION OF THE BACILLUS ISOLATED FROM THE ENSILAGE TO *B. BOTULINUS*

The biological and pathological characters of the bacillus (laboratory index ss) isolated from the McLean ensilage suggested the possibility of securing an efficacious antitoxic serum by injecting sublethal doses into a goat. Serum (A-1) from a goat rendered immune to the bacillus (ss) isolated from the McLean ensilage was administered intraperitoneally to three guinea-pigs in doses of 0.5 mil, 1 mil and 1.5 mils respectively, followed in twenty-four hours by five times the lethal amount of broth cul-

ture of the bacillus (ss) administered by the mouth. The antitoxic serum (A-1) apparently failed to protect the pig receiving 0.5 mil. The other two pigs remained healthy. A control pig receiving a similar amount of the toxin (ss) died in approximately eighteen hours. In a duplicate test similar results were obtained, suggesting that the antitoxic serum (A-1) did not afford complete protection in 0.5 mil doses against five times the lethal dose of (ss) toxin, while 1 mil and 1.5 mils of the serum apparently did afford protection (see chart 1, and fig. 1).

CHART 1

Potency test of goat serum (A-1) immune to bacillus (ss) from ensilage

GUINEA-PIG		INJECTION OF GOAT SERUM (A-1) IMMUNE TO BACILLUS FROM ENSILAGE	ADMINISTRATION OF BROTH CULTURE OF BACILLUS (SS) FROM ENSILAGE	RESULTS
Number	Weight			
	grams			
1	200	0.5 mil June 18, 1917	0.1 mil June 19, 1917	Dead 7 a.m. June 28, 1917
2	250	1.0 mil June 18, 1917	0.1 mil June 19, 1917	Released July 2, 1917
3	250	1.5 mils June 18, 1917	0.1 mil June 19, 1917	Released July 2, 1917
4	300	0	0.1 mil June 19, 1917	Dead 7 a.m. June 20, 1917



FIG. 1. POTENCY TEST OF GOAT SERUM (A-1) IMMUNE TO BACILLUS (SS) ISOLATED FROM MCLEAN ENSILAGE

Control dead in approximately eighteen hours (see table 1).

Following the drawing of serum (A-1) sublethal doses of toxin were administered to the goat, after which an antitoxic serum (B-1 and C-1) was obtained which apparently protected guinea-pigs in doses of 0.5 mil administered intraperitoneally against 0.1 mil of the homologous toxin (see chart 2). Goat serum B-1 and C-1 immune to the bacillus (ss) isolated from the ensilage, administered intraperitoneally in amounts of 0.5 mil, 1 mil and 1.5 mils apparently provided protection to guinea-pigs against a fatal artificial infection of *B. botulinus* administered by the

CHART 2

Potency test of goat serum (B-1 and C-1) immune to bacillus (ss) from ensilage

Number	Weight grams	INJECTION OF GOAT SERUM OR LETHAL DOSE IMMUNE TO BACILLUS (SS) FROM ENSILAGE	ADMINISTRATION OF BROTH CULTURE OF BACILLUS (SS) FROM ENSILAGE	RESULTS	
1	450	0.5 mil July 10, 1917	0.1 mil July 11, 1917	Healthy	Released July 25, 1917
2	350	1.0 mil July 10, 1917	0.1 mil July 11, 1917	Healthy	Released July 25, 1917
3	400	1.5 mils July 10, 1917	0.1 mil July 11, 1917	Healthy	Released July 25, 1917
4	550	0	0.1 mil July 11, 1917	Dead 7 a.m.	July 12, 1917

mouth twenty-four hours later. An unprotected pig succumbed in twenty hours (see chart 3).

Mule serum immune to *B. botulinus*, protected guinea-pigs against five times the lethal amount of broth culture of the bacillus (ss) administered by the mouth. A composite sample of antitoxic serum, consisting of series A, B, C, drawings was injected intraperitoneally into three guinea-pigs in doses of 0.5 mil, 1 mil, and 1.5 mils, respectively. At the end of twenty-four hours each treated pig was given per os, 0.1 mil broth culture of the bacillus (ss). At the same time an untreated pig received a similar amount of broth culture of the bacillus (ss). The pigs receiving the serum remained apparently healthy while the con-

CHART 3

Serological relation of serum immune to bacillus (ss) from ensilage to B. botulinus

GUINEA-PIG		INJECTION OF GOAT SERUM (B-1 AND C-1) IMMUNE TO BACILLUS (SS) FROM ENSILAGE	ADMINISTRATION OF B. BOTULINUS *IN BROTH CULTURE	RESULTS
Number	Weight			
	grams			
1	400	0.5 mil July 2, 1917	0.1 mil July 3, 1917	Healthy. Released July 17, 1917
2	500	1.0 mil July 2, 1917	0.1 mil July 3, 1917	Healthy. Released July 17, 1917
3	550	1.5 mils July 2, 1917	0.1 mil July 3, 1917	Healthy. Released July 17, 1917
4	500	0	0.1 mil July 3, 1917	Dead 7 a.m. July 4, 1917

* *B. botulinus* N.B.S. strain received from Dr. J. S. Buckley of the Pathological Laboratory, Bureau of Animal Industry, Washington, D. C.

trol pig succumbed in approximately twenty-one hours (see chart 4).

Serum immune to *B. botulinus*, administered intraperitoneally, apparently protected guinea-pigs against many times a lethal amount of the sterile filtered broth culture, or of the unfiltered broth culture of the bacillus (ss) isolated from the ensilage.

CHART 4

Serological relation of serum immune to B. botulinus to bacillus (ss) from ensilage

GUINEA-PIG		INJECTION OF BOTULINUS ANTITOXIN MULE SERUM— A, B AND C	ADMINISTRATION OF BROTH CULTURE OF BACILLUS (SS) FROM ENSILAGE	RESULTS
Number	Weight			
	grams			
1	300	0.5 mil May 19, 1917	0.1 mil May 20, 1917	Healthy. Released May 30, 1917
2	350	1.0 mil May 19, 1917	0.1 mil May 20, 1917	Healthy. Released May 30, 1917
3	350	1.5 mils May 19, 1917	0.1 mil May 20, 1917	Healthy. Released May 30, 1917
4	350	0	0.1 mil May 20, 1917	Dead 7 a.m. May 21, 1917

An animal was subjected to several sublethal doses of *B. botulinus* toxin and at intervals of time serum was drawn from the jugular vein. A composite sample of antitoxic mule serum, series E and F, was injected intraperitoneally into three guinea-pigs in doses of 0.5 mil, 1 mil and 1.5 mils, respectively. Twenty-two hours later these animals received by the mouth 0.1 mil broth filtrate of the bacillus (ss) isolated from the ensilage. A control pig receiving an equal amount of the filtrate per os succumbed in twenty hours, while the serum treated pigs remained apparently healthy and were released after a period of twenty days (see chart 5).

CHART 5

Serological relation of serum immune to B. botulinus to sterile filtered broth culture of bacillus (ss) from ensilage

GUINEA-PIG		INJECTION OF BOTULINUS ANTITOXIN MULE SERUM E AND F	ADMINISTRATION OF FIL- TERED BROTH CULTURE OF BACILLUS (ss) FROM ENSILAGE	RESULTS
Number	Weight grams			
1	350	0.5 mil May 30, 1917	0.1 mil May 31, 1917	Healthy. Released June 20, 1917
2	350	1.0 mil May 30, 1917	0.1 mil May 31, 1917	Healthy. Released June 20, 1917
3	300	1.5 mils May 30, 1917	0.1 mil May 31, 1917	Healthy. Released June 20, 1917
4	340	0	0.1 mil May 31, 1917	Dead 1 p.m. June 1, 1917

It has been noted that goat serum immune to the bacillus (ss) protected guinea-pigs against 0.1 mil broth culture of the homologous strain per os, and also against 0.1 mil broth culture of *B. botulinus* per os. (Goat serum (J) highly immune to *B. botulinus*, injected intraperitoneally into three guinea-pigs in doses of 1 mil, 1.5 mils and 2 mils respectively, apparently provided protection against ten times the lethal amount of the broth culture per os of the bacillus (ss). A control pig receiving a similar amount of the broth culture succumbed in approximately twenty-two hours (see chart 6 and fig. 2).

CHART 6

Serological relation of serum immune to B. botulinus to bacillus (ss) from ensilage

Number	GUINEA-PIG		INJECTION OF BOTULINUS ANTITOXIN (GOAT SERUM J)	ADMINISTRATION OF BROTH CULTURE OF BACILLUS (SS) FROM ENSILAGE	RESULTS
	Weight	grams			
1	600		0.5 ml May 12, 1917	0.2 ml May 13, 1917	Healthy. Released May 28, 1917
2	550		1.0 ml May 12, 1917	0.2 ml May 13, 1917	Healthy. Released May 28, 1917
3	950		2.0 mls May 12, 1917	0.2 ml May 13, 1917	Healthy. Released May 28, 1917
4	500		0	0.2 ml May 13, 1917	Dead 7 a.m. May 11, 1917



FIG. 2. SEROLOGICAL RELATION OF BOTULISM ANTITOXIN (GOAT SERUM J) TO BACILLUS (SS) ISOLATED FROM ENSILAGE

The antitoxin apparently protected the three treated pigs (see table 6).

Similar serological tests were also made upon horses. Blemished and unsound horses were employed in this work, but all animals were serviceable and in good health. Horses 1017 and 1018 received at 4 p.m. May 15, 1917, intravenous injections (40 mls) of mule serum, series E and F, immune to *B. botulinus*. The following day these horses and a control horse, no. 119, were each allowed to ingest 2 mls broth culture of the bacillus (ss) in 1000 grams of wholesome oats (fig. 3). These animals were then supplied with wholesome feed and water each day. On May 19, horse 119 prehended and masticated feed awkwardly, accom-

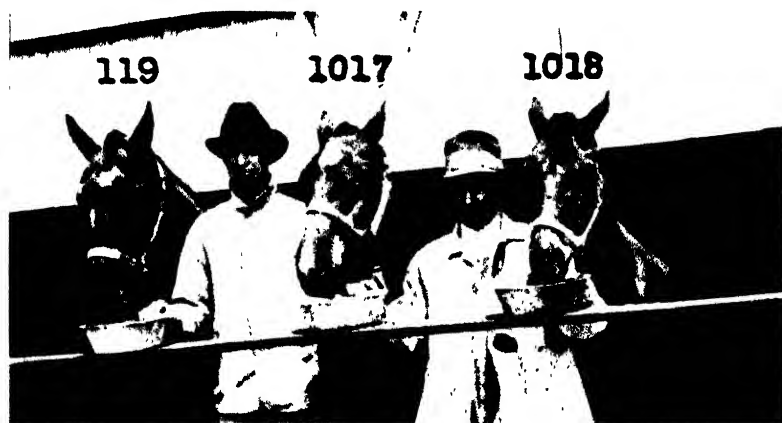


FIG. 3. HORSES 1017 AND 1018 RECEIVED PROPHYLACTIC DOSE OF SERUM IMMUNE TO *B. BOTULINUS*, MAY 15, 1917

The following day these horses and a control, No. 119, each received on wholesome feed 2 mls broth culture of the bacillus (ss) isolated from the ensilage.

panied by slight ptialism. On May 20, a drawn appearance of the flank, profuse ptialism, muscular weakness, paresis of the pharynx and a stupid, listless attitude were observed, and at 1 p.m. the animal became decumbent. The photograph for figure 4 was made at 2.30 p.m. May 20. In a decumbent position the animal moved the feet violently and suffered from marked dyspnoea. On May 21, decumbency continued, accompanied by marked dyspnoea. The tongue was pendulous and protruding.

feed was refused, and an audible clicking sound was noted in the pharynx. May 22, decumbency continued, ptyalism, mucous discharge from the nostrils, marasmus and enuresis were observed. May 23, death occurred at 7 a.m. Post-mortem examination revealed gross lesions resembling those found in animals naturally afflicted with forage poisoning. Horses 1017 and 1018 appeared dull and exhibited mild transitory symptoms on May 19 and 20, which soon subsided and the animals remained apparently healthy (see chart 7).



FIG. 4. SAME ANIMALS, SHOWING NO. 119 IN A DECUMBENT POSITION

Photographed May 20, 1917 (see table 7).

Agglutination. The agglutinins present in sera highly immune to *B. botulinus* proved active to the homologous strain and to the bacillus isolated from the ensilage in question. Serum immune to the organism (ss) contained agglutinins active to the homologous strain and to *B. botulinus* (see chart 8). Sera immune to a similar bacillus isolated from an oat hay, to a bacillus isolated from the caecum of a horse following death from drinking water in which the above oat hay was immersed, and to a bacillus

CHART 7

Serological relation of serum immune to B. botulinus to the Bacillus (ss) isolated from the ensilage

HORSE		INJECTION OF BOTULINUS ANTITOXIN MULE SERUM E AND F	ADMINISTRATION OF BROTH CULTURE OF BACILLUS (ss) FROM ENSILAGE	RESULTS
Number	Weight lbs.			
1017	800	40 mls May 15, 1917	2 mls May 16, 1917	Healthy. Released July 2, 1917
1018	830	40 mls May 15, 1917	2 mls May 16, 1917	Healthy. Released July 2, 1917
119	820	0	2 mls May 16, 1917	Dead 7 a.m. May 23, 1917

CHART 8

Agglutination test of B. botulinus (NBS) immune sera from other strains of B. botulinus, B. coli, B. chauvæi and normal sera of sheep, guinea pig, and rabbit

	DILUTION						Control
	1-100	1-200	1-500	1-1000	1-2000	1-5000	
Horse serum immune to NBS strain	++++	++++	++++	+++	+++	++	-
Mule serum immune to NBS strain	++++	++++	++++	++	++	+	-
Horse serum immune to GO6 strain	++++	++++	++++	++++	+++	+	-
Horse serum immune to C91 strain	++++	++++	++++	++	+	-	-
Goat serum immune to SS strain	++++	++++	++++	+++	+++	++	-
Sheep serum immune to GS strain	++++	++++	++++	+++	+++	++	-
Serum immune to <i>B. coli</i> species	++	-	-	-	-	-	-
Serum immune to <i>B. chauvæi</i>	+	-	-	-	-	-	-
*Normal sheep serum....	++++	+++	++	-	-	-	-
*Normal guinea-pig serum	-	-	-	-	-	-	-
*Normal rabbit serum...	-	-	-	-	-	-	-

*"Normal" is used to indicate that the animal had not been artificially treated with immune sera or sublethal doses of toxin.

isolated from an ensilage in a remote outbreak of forage poisoning, also contained agglutinins which were active to *B. botulinus* and to the bacillus (ss) isolated from the ensilage (see chart 9). Sera immune to *B. coli*-like organisms and to *B. chauvæi* showed slight and inconsistent agglutinating potency in dilutions of 1-100 to *B. botulinus* and the organism (ss) isolated from the ensilage in

CHART 9

Agglutination test of Bacillus (ss) isolated from ensilage with immune sera from other strains of B. botulinus, B. coli, B. chauvæi, and normal sera of sheep, guinea pig and rabbit

	DILUTION						Control
	1-100	1-200	1-500	1-1000	1-2000	1-5000	
Horse serum immune to NBS strain.....	++++	++++	++++	++++	+++	++	-
Mule serum immune to NBS strain.....	++++	++++	++++	++++	+++	+++	-
Horse serum immune to GO6 strain.....	++++	++++	++++	+++	++	++	-
Horse serum immune to C91 strain.....	++++	++++	++++	++++	+++	++	-
Goat serum immune to SS strain.....	++++	++++	++++	++++	++++	+++	-
Sheep serum immune to GS strain.....	++++	++++	++++	++++	++++	++	-
Serum immune to <i>B. coli</i> species.....	+++	++	-	-	-	-	-
Serum immune to <i>B. chauvæi</i>	++	-	-	-	-	-	-
Normal sheep serum...	+++	+	-	-	-	-	-
Normal guinea-pig serum.....	-	-	-	-	-	-	-
Normal rabbit serum.	-	-	-	-	-	-	-

question. The sera of the untreated guinea-pig, rabbit and horse showed no agglutinating potency to *B. botulinus* nor to the bacillus (ss) isolated from the ensilage. In one instance serum from an untreated sheep showed mild agglutinating potency to *B. botulinus* in a dilution of 1-200. The presence of agglutinins in the serum of this animal in low dilutions was repeatedly observed (see chart 8 and 9).

DISCUSSION

The extent of forage poisoning in animals resulting from toxin-producing organisms resembling *B. botulinus* is not known, nor is the method by which ensilage and other animal feeds become contaminated well understood. A limited observation suggests that certain conditions in nature are favorable for the development of this type of organism on masses of feed. In this connection it is noted that the three animal feeds from which *B. botulinus* or closely allied organisms have been isolated were stored en masse from two to six months before the contaminated portions were encountered in the feeding operations. Two contaminated feeds in our observations were obtained from silos, while a third, an oat hay, was found stored in a frame barn. The ability of *B. botulinus* and the bacillus (ss) isolated from the McLean ensilage to develop under aerobic conditions with *Fusarium* sp., and also in acid medium, suggests that such organisms might survive in nature under varying conditions. Jordan (1917) states that practically all cases of botulism in human beings have been caused by food that has received some sort of preliminary treatment, such as smoking, canning or pickling. A majority of animal feeds are harvested, cured and stored in bulk for winter feeding, but it is very doubtful if all outbreaks of forage poisoning are associated with stored feed as the clinical disease has been observed in animals on pasture. Outbreaks of botulism in man have been recorded following the consumption of vegetable and fruit products (Dickson, 1916), and the name *botulism* has thus lost its original significance (*botulus* = sausage) as related to the disease in the human family. Even less appropriate is the term *botulism* as applied to forage poisoning or cerebrospinal meningitis in animals. The term *forage poisoning* appears to be without objection as applied to this type of clinical disease in domestic animals.

The presence on animal feeds of anaerobic bacilli capable of engendering a fatal intoxication when propagated artificially is suggestive of the possible prophylactic or curative value of anti-toxic serum in sporadic outbreaks of forage poisoning. In emer-

gency it might be employed as a possible aid to suppress the economic loss from this disease. The value of forage poisoning antitoxin (botulinus antitoxin) in outbreaks of forage poisoning in animals cannot be relied upon until a larger amount of dependable field data is available, but it seems evident from our observations that certain types of this disease may eventually prove amenable to the serum treatment. The use of polyvalent antitoxic serum in outbreaks of forage poisoning, followed by careful observations, might also contribute information relative to the prevalence of *B. botulinus* or closely allied anaerobic toxin-producing organisms encountered in fatal diseases of animals recognized as forage poisoning or cerebrospinal meningitis.

SUMMARY

1. An anaerobic, spore-bearing bacillus (ss) isolated from a corn ensilage, proved to be responsible for an outbreak of forage poisoning at Ottawa, Illinois, engendered clinico-anatomic forage poisoning in a mule following the ingestion of 2 mls of the sterile filtrate broth culture. 0.02 and 0.03 mil broth culture per os generally proved fatal in guinea-pigs in twenty-two to eighty-four hours.

2. A goat injected with sublethal doses of broth culture of the bacillus (ss) isolated from the ensilage developed an antitoxic serum which injected intraperitoneally apparently protected guinea-pigs against a fatal amount of the homologous toxin per os. The protective nature of the goat serum was also observed in guinea-pigs receiving fatal amounts of *B. botulinus* per os.

3. *B. botulinus* antitoxic serum prepared from a mule and injected intraperitoneally into guinea-pigs apparently provided a protection against 10 times the lethal amount of the culture filtrate of the bacillus (ss) isolated from the ensilage and against five times the lethal dose of the unfiltered broth culture.

4. Two horses ingested in wholesome feed 2 cc. broth culture of the bacillus (ss) isolated from the ensilage and were apparently protected by an intravenous injection of botulinus antitoxic serum. A control horse receiving a similar amount of the unfil-

tered broth culture manifested clinical symptoms of forage poisoning and died. Post-mortem examination revealed gross anatomic lesions analagous to some of the gross lesions observed in animals naturally afflicted with forage poisoning.

5. Serum immune to *B. botulinus* possessed a positive agglutinating potency toward the homologous strain, as well as toward the bacillus (ss) isolated from the ensilage. The agglutinins present in serum immune to the bacillus (ss) isolated from the ensilage were active against *B. botulinus* as well as against the homologous strain.

6. The cultural, morphological, pathological, and serological relation observed between *B. botulinus* and the bacillus isolated from the ensilage in preliminary experiments are suggestive of the possible etiologic significance of *B. botulinus* or closely allied anaerobes in some outbreaks of forage poisoning or cerebrospinal meningitis in horses.

The authors wish to acknowledge the coöperation of Professors Rusk and Grindley of the Illinois Agricultural Experiment Station in affording us an opportunity to study the outbreak of forage poisoning on the McClean Farm and in furnishing us samples of the silage for the investigations here reported.

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AEROBIC SPORE-BEARING BACTERIA IN THE INTESTINAL TRACT OF CHILDREN

MARJORIE DIETZ BATCHELOR

*From the Bacteriological Laboratory and the Harriet Lane Home, Johns Hopkins
University*

Received for publication January 14, 1918

During the last three years a bacteriological investigation of the dejecta of children has been undertaken to determine whether changes in the intestinal flora can be correlated with changes in diet, the results of this investigation being published elsewhere. In the course of this work it became necessary to make a systematic study of the aerobic spore-bearing bacteria which were isolated from the various cases, and the results obtained seem of sufficient importance to merit separate consideration.

Smears made from the dejecta of children when stained by Gram's method, reveal large Gram-positive bacteria which differ markedly in size and shape from the Gram-negative organisms of the intestine. In adults and in many children, they form a relatively unimportant constituent of the faecal flora. In other children, however, especially when a diet of protein milk or buttermilk has been administered, these large Gram-positive organisms are much increased in number and form a considerable proportion of the organisms found on bacterioscopic examination. Together with these forms, spores of various sizes and shapes may be detected; and the large Gram-positive organism are usually, in our opinion, aerobic spore-bearers. Ordinary agar and gelatin plates made from the dejecta reveal only the small opalescent colonies of *Bacillus coli* or *Bacillus alkaligenes* or the large viscid colonies of *Bacillus lactis-aerogenes*, all of which are Gram-negative. If an emulsion of the dejecta be made in broth and heated to 80°C. for twenty minutes and cultures be taken from this at once, or after twenty-four hours in-

cubation, aerobic spore-bearing bacteria, usually Gram-positive, develop in great profusion. The morphology of the various species, particularly that of the members of the mesentericus, cereus and megatherium groups, and the size and shape of the spores are so characteristic and correspond so closely to the morphology of the Gram-positive bacteria and the spores seen in the dejecta as to leave no reasonable doubt of the identity of the two groups. Occasionally when the Gram-positive organisms are in great abundance in the contents of the intestine they appear on plates made directly from the dejecta and here the correspondence between the smears and the cultures is still more striking.

We have now examined the dejecta of over fifty children with the special object of isolating and identifying the aerobic spore-bearers. Several hundred cultures have been studied, the various species being found in the different cases in about the following proportion:

<i>Aerobic spore-bearing bacteria in the dejecta of children</i>	
<i>Species</i>	<i>Number of cases</i>
<i>Bacillus cereus</i>	32
<i>Bacillus albolactis</i>	17
<i>Bacillus pseudotetanus</i>	9
<i>Bacillus mesentericus</i>	6
<i>Bacillus subtilis</i>	6
<i>Bacillus petasites</i>	2
<i>Bacillus vulgatus</i>	2

From the above table it may be seen that the aerobic spore-bearing bacteria of the intestinal tract in children are the same which have been previously established as the predominant organisms of our environment in Baltimore, in soil, dust, water and milk. It is interesting to note that *Bacillus subtilis* is somewhat rare in the intestines, contrary to the usual statements. This is probably to be explained by the fact that only those organisms corresponding to the *Bacillus subtilis* of Cohn as described by Gottheil (1901), Chester (1901), and Lawrence and Ford (1916), are included in this group, whereas the large Gram-positive elements with oval spores, formerly called *Bacillus subtilis* by many writers, are now referred to the species *Bacillus cereus* and

Bacillus albolactus. The relative frequency of *Bacillus pseudotetanus* should also be noted. This is an aerobic species which is much like the tetanus bacillus in morphology and which produces a round spore in a terminal or sub-terminal position in the vegetative rod. It also resembles *Bacillus putrificus-coli* of Bienstock, one of the putrefactive anaerobes. Since *Bacillus pseudotetanus* is so common, the finding in the dejecta of organisms with round terminal spores, can not in the absence of positive cultures, be regarded as satisfactory evidence that the individuals are harboring the tetanus bacillus; nor can the presence of organisms with this morphology be taken to indicate excessive anaerobic putrefaction. In addition to these well-recognized species, cultures were isolated on five occasions which could not be identified with any previously described in the literature nor with any of the strains kept in the laboratory for purposes of comparison. These organisms have, therefore, been described as new species as follows:

Bacillus badius nov. sp.

This organism is characterized by the beautiful arborescent colonies which it produces on agar plates and in agar stab cultures. These colonies are not unlike those of *Bacillus mycoides* and *Bacillus prausnitzii* of the mycoides group to which also *Bacillus adhaerens* of Laubach has been referred (1916). It differs from these organisms in its cultural reactions and in its production of a brown pigment, but may be placed in the same group. It has been found in one stool.

Morphology. Vegetative rods long with homogeneous protoplasm and round ends, varying in length from 2.35 to 4.5 microns in twenty-four hour cultures on plain agar (fig. 1). The shorter forms predominate in older cultures. Width about 0.75 micron. On glucose agar the organisms are somewhat swollen (fig. 2). Rarely short chains are formed. Whip forms not seen.

Motility. Active motility in young cultures.

Staining reactions. Gram-positive.

Spore formation. Spores begin to form by the first day on glucose agar but only after four to five days on plain agar. They are oval in shape, subterminal in position. As the sporulating rods degenerate, tags of protoplasm are left, sometimes at one end and sometimes at

both ends of the spore. They measure 1.125 by 1.875 microns. Older spores become free from protoplasmic tags and assume an oval contour with a tendency to squaring of the ends. Such naked spores measure about 0.75 by 1.5 microns (fig. 3).

Agar slant. Rapidly spreading glistening moist growth, buff-colored in young cultures with areas of brown pigmentation. At the end of forty-eight hours the entire growth is brown. The surface remains moist and glistening and the growth is easily scraped from the agar. Later the agar itself becomes pigmented.

Agar stab. Slight growth along line of inoculation definitely arborescent. Surface growth at point of puncture spreading and abundant.

Agar colonies. Buff opaque moist colonies showing great irregularity in size and shape. The edges are fuzzy with beautiful arborizations. Under low power, the colonies are reticulated with fine extensive radial outgrowths.

Litmus glucose agar. Rapidly spreading dry buff-colored growth easily scraped from the medium. No acid. Agar gradually becomes dark blue.

Litmus glucose agar colonies. Colonies similar to those on plain agar with somewhat accentuated arborizations. Dirty white to pale brown in color. No acidity.

Gelatin stab. Slow growth along line of inoculation with rapid cup-shaped surface liquefaction. The liquid gelatin becomes definitely brown.

Gelatin colonies. Irregular brown colonies. No definite arborization. The edges of the colonies are fuzzy and their texture resembles cotton. Rapid liquefaction.

Broth. Rapid production of dense turbidity. No scum. No precipitate. Medium becomes very brown.

Peptone. Fairly rapid growth without scum or precipitate. Medium becomes brown in color.

Potato. Faint dry brown growth.

Litmus milk. Gradual decolorization of the litmus and clearing of the proteins in the milk. Digestion complete in about a week when the fat at the surface becomes brown from the adsorption of the pigment.

Blood serum. Dry buff growth with some tendency to a beaded appearance. It becomes brown in two to three days. Some softening of the serum which gradually dried without definite liquefaction.

Fermentation Tubes. Glucose: Turbidity in bulb and neck. No scum or precipitate. Reaction neutral.

Sucrose: Turbidity in bowl and neck. No scum. No precipitate. Reaction neutral.

Lactose: Turbidity in bowl. No scum. No precipitate. Reaction neutral.

*Thermal death point.*¹ Spores in broth emulsion survive a pressure of $2\frac{1}{2}$ pounds in the autoclave but are destroyed by 5 pounds pressure. They survive fifteen minutes steaming in the Arnold but are destroyed by thirty minutes exposure.

Bacillus fusus nov. sp.

This organism has been found but once in a specimen of stool planted directly in milk. Morphologically it is similar to the species recently described by Ford (1916), *Bacillus centrosporus*. It differs from it in its reaction to Gram's stain, being Gram-positive, and in certain fundamental cultural features.

Morphology. In twenty-four hours cultures on plain agar the vegetative rods lie singly, are thin and delicate with homogeneous protoplasm (fig. 4). They vary greatly in size and shape because of the early sporulation. They are usually regular in outline with round ends and measure 2.25 to 4.5 microns in length by 0.75 micron in thickness. The longer forms predominate. On glucose agar the rods are thicker measuring 1.125 microns and show many long forms, 4 to 6 microns, with whip forms 12 to 20 microns in length (fig. 5). In older glucose agar cultures the whip forms are much longer, often running across the field of the microscope or lying wound up in long coils. They are usually thinner than the short rods, having an average width of about 0.75 microns. In cultures four to five days old involution forms begin to appear, as small heavily staining globular masses measuring 1.5 to 1.875 microns in width by 2.25 to 3.75 microns in length. Shadow forms are common on plain agar.

Motility. Active progressive motility in young broth cultures.

Staining reactions. Gram-positive.

Spore formation. Sporulation begins on plain agar in twenty-four hours but seldom occurs in glucose agar where the involution forms pre-

¹ In determining the thermal death point in the autoclave the pressure is successively raised to the various points indicated, $2\frac{1}{2}$ pounds, 5 pounds, etc., after which the gas is turned off at once and the autoclave allowed to cool. The organisms are thus exposed for only a brief time, usually less than a minute, to the various pressures.

dominate. The spores form in the center of the rods which become pointed as the spore develops (fig. 4). The rods thus take on a spindle-shape (fig. 6). The sporangia are often irregularly swollen with one or both ends pointed. As the spores mature the organisms are bulged, becoming very thick centrally because of the size of the spore. The free spores are large compared with the rods, oval and measure 1.125 by 2.25 microns. They do not retain protoplasmic tags but are provided with a rather thick outer rim of ectoplasm (exine). Great variation is seen in the size and shape of the spores. Some remain oval, others are longer, measuring 1.125 by 3 microns. Others are swollen, measuring 1.875 by 2.25 microns. At times the sides are flattened and the spores become reniform.

Agar slant. Thin white beaded growth never becoming very abundant. Growth at times adherent to the medium.

Agar stab. Faint growth along line of inoculation with a slight bead-like knob at the point of puncture.

Agar colonies. Small pin-point white colonies, smooth and round, with dark centers and pale peripheries. Under low magnification, homogeneous with complete edges.

Litmus glucose agar. Growth more abundant than on plain agar, beaded, without tendency to spread. Easily scraped from the medium. Gradually increasing and persistent acidity.

Litmus glucose agar colonies. Small round white colonies, larger than on plain agar. Under low power usually homogeneous with complete edges but the colonies may show fine reticulation.

Gelatin. Faint growth along line of inoculation. No liquefaction.

Gelatin colonies. Small, irregular or round pale colonies with well defined edges. No liquefaction.

Broth. Slight turbidity and granular precipitate. No scum.

Peptone. Slight turbidity and granular precipitate. No scum.

Potato. Moist, creamy rather abundant growth, at times pale brown in color.

Litmus milk. Gradual reduction of the litmus without digestion of the proteins or coagulation.

Blood serum. Very faint, dry and hardly visible growth.

Fermentation tubes. Glucose: Faint turbidity with a slight granular precipitate in the bowl and neck. No scum. Reaction acid.

Sucrose: Faint turbidity, slight precipitate. No scum. Reaction acid.

Lactose: Faint turbidity with fine granular precipitate. No scum. Reaction acid.

Thermal death point. Spores survive 5 pounds pressure in the autoclave but are destroyed by 7 pounds. They resist steaming in the Arnold for fifteen minutes but succumb to thirty minutes exposure.

Bacillus tritus nov. sp.

This organism has been placed in the simplex cohaerens group chiefly because of the size of the vegetative rods and the method of sporulation. It does not have all of the cultural characteristics of any of the members of this group, and must therefore be regarded as a new species. It has been isolated on but one occasion.

Morphology. The morphology is best observed in forty-eight hour agar culture where the organisms usually lie singly. They are regular in outline, with homogeneous protoplasm and round ends. They measure usually 0.75 by 3.75 microns (fig. 7). Often short almost oval forms occur. On glucose agar the rods are larger averaging 0.75 by 5.25 microns. Whip formation occurs on plain agar and is common on glucose agar (fig. 8). Many of these whips measure 75 microns in length.

Motility. Active progressive motility in young cultures.

Staining reactions. Gram-positive.

Spore formation. The spores are smaller than the vegetative rods. They form early, often appearing in twenty-four hours on plain and glucose agar. They are oval, measuring 1.125 by 1.5 microns and subterminal in position in the vegetative rods. The young spores retain protoplasmic tags of various lengths. As the spores mature these tags are lost, the spores become oval and then measure 0.75 by 1.125 microns (fig. 9).

Agar slant. Thick creamy moist glistening growth made up in young cultures of confluent colonies, about 0.5 mm. in diameter. These gradually coalesce. Older cultures are buff in color and have a tallow-like consistency.

Agar stab. Faint growth along line of inoculation. Translucent buff growth at point of puncture.

Agar colonies. Moist opaque round and irregular surface colonies with dark centers and rather compact but fuzzy peripheries. Deep colonies clear-cut, round or elliptical with occasional fuzzy outlines. Under low power the colonies are reticulated, particularly in the peripheral zones.

Litmus glucose agar slant. Thin dry beaded growth in twenty-four hours with a faint acidity which does not persist.

Litmus glucose agar colonies. Small white almost pin-point surface colonies. Under low power pale, round or irregular with complete edges. Deep colonies punctiform.

Gelatin stab. Faint growth along line of inoculation. No liquefaction.

Gelatin colonies. Rather large irregular brown homogeneous colonies with circumscribed edges. No liquefaction.

Broth. Turbidity and cloudy precipitate which later becomes granular. Fragile pellicle.

Peptone. Faint turbidity and precipitate. No pellicle.

Potato. Faint dry brown growth.

Litmus milk. Faint growth in milk with practically no change in the medium. No acidity. No coagulation.

Blood serum. Shiny wax-like buff growth becoming pale brown. No liquefaction.

Fermentation tubes. Glucose: Faint turbidity in the bulb. Slight sediment. No scum. Reaction neutral.

Sucrose: Appearance the same. Reaction neutral.

Lactose: Appearance the same. Reaction neutral.

Thermal death point. Spores destroyed by $7\frac{1}{2}$ pounds pressure in the autoclave. They survive thirty minutes steaming in the Arnold but are destroyed by exposure for forty-five minutes.

Bacillus lautus nov. sp.

This organism has been placed provisionally in the simplex-cohaerens group, partly because of its morphology and spore-formation and partly because of its cultural characteristics. It differs from the cultures of *Bacillus simplex* and *Bacillus cohaerens* now in the laboratory and from *Bacillus agri*, the species recently described by Laubach and Rice (1916), which is also included in this group. It has been isolated on but one occasion.

Morphology. In twenty-four and forty-eight hour agar cultures the vegetative rods are homogeneous with rounded ends. They vary greatly in size and shape and usually measure 1.5 microns in thickness by 2.25 to 4.5 microns in length (fig. 10). Shadow forms appear early with irregular aggregations of cytoplasm. They are larger than the vegetative rods, measuring 1.875 by 5.25 to 6 microns. Whip forms also appear early (fig. 10) and are very common in old cultures. Involution forms are frequent in old agar growths taking the stain deeply

and measuring 2.25 by 3 microns. On glucose agar the organisms are thicker and often shorter, measuring 1.875 by 2.25 microns (fig. 11), but longer forms predominate. Involution forms are very numerous on old glucose agar.

Motility. Actively motile in young cultures.

Staining reactions. Gram-positive.

Spore formation. Spores are found early on plain agar but later on glucose agar where they seldom appear before ninety-six hours. They are subterminal, wider than the organism from which they spring, and measure about 1.875 by 3 microns. As they mature they lose their protoplasmic rims and become more oval, measuring usually 1.125 by 2.25 microns (fig. 12).

Agar slant. Very thin dry transparent growth without tendency to spread.

Agar stab. Very faint growth along line of puncture with a meager surface growth at point of inoculation.

Agar colonies. Tiny white colonies round and irregular. Under low power granular with complete edges.

Litmus glucose agar. Fairly heavy dry beaded growth. Reaction acid.

Litmus glucose agar colonies. Thin moist irregular and round colonies with dark centers and lighter peripheries. Under low power homogeneous translucent with serrate edges. Acidity marked.

Gelatin stab. Faint growth along line of inoculation. No liquefaction.

Gelatin colonies. Small white colonies with hazy irregular outlines. No liquefaction.

Broth. Early turbidity and late production of a hazy rather flocculent precipitate.

Peptone. Faint growth, slight turbidity, no precipitate.

Potato. Faint moist pale-brown growth.

Litmus milk. Rapid reduction of the litmus, gradual development of acidity which persists. No coagulation. No peptonization.

Blood serum. Diffuse thin pale-brown growth with areas of yellow pigmentation. This develops into a rather dirty brown growth in seven to eight days. No liquefaction.

Fermentation tubes. Glucose: Turbidity in open bulb. No precipitate. No scum. Reaction acid.

Sucrose: Turbidity in bulb. No precipitate. No scum. Reaction acid.

Lactose: Turbidity in bulb. No precipitate. No scum. Reaction acid.

Thermal death point. Spores destroyed in the autoclave at a pressure of 10 pounds. Survive $7\frac{1}{2}$ pounds pressure. Destroyed by live steam in the Arnold sterilizer in thirty minutes.

Bacillus flexus nov. sp.

This organism has been placed in the megatherium group because of the large size of the vegetative rods and the early production of swollen bodies with an aggregation of the cytoplasm in globular masses. In old cultures there is a marked tendency to the development of curved or spiral forms such as are seen in *Bacillus graveolens*. The organism however differs in its cultural reactions from this species and from the other members of the megatherium group described in the literature. It has been encountered but once.

Morphology. On plain agar in young cultures the vegetative rods are large, with squared ends, measuring 1.5 to 1.825 microns in width and 2.25 to 4.5 microns in length with many of the forms equalling 5.25 microns in length (fig. 13). Chain formation is frequent. The protoplasm is homogeneous in the younger elements but rapidly becomes granular as the organisms mature. On glucose agar the larger forms predominate. On both plain agar and glucose agar the rods swell, the protoplasm segments and the elements characteristic of *Bacillus megatherium* and *Bacillus petasites* are seen in great profusion.

These forms measure 1.5 to 2.2 by 4.5 to 6 microns, have little of the usual cytoplasm left but in its place a mass of large highly refractile globular bodies (fig. 14). These are numerous in the various organisms as many as six or eight having been observed in a single bacillus. Shadow forms develop early, often in twenty-four hours. They measure 1.125 to 1.5 by 4 to 10 microns. In older cultures these shadow forms with protoplasmic remnants are very common (fig. 15). In such cultures long chains of organisms twisted on themselves are seen in abundance while the individual elements are often bent or curved and assume bizarre shapes (fig. 15). Whip forms are also common (fig. 16).

Motility. Active progressive motility in young cultures.

Staining reaction. Gram-positive.

Spore formation. Spores are formed early on glucose agar appearing in forty-eight hours but are never plentiful on plain agar. They are

usually terminal or subterminal and resemble megatherium spores. They are wider than the vegetative rod which bulges on sporulation. The free spores are round, measuring 1.5 by 1.5 microns, or oval, measuring 1.125 by 1.5 microns.

Agar slant. Heavy tallow-like growth along the line of inoculation, gradually becoming pale yellowish-brown in color.

Agar stab. Slight development along line of inoculation and tallow-like surface growth.

Agar colonies. Rather profuse dirty-white tallow-like colonies, with circumscribed edges. They vary greatly in shape, some being round and others very irregular. Under low power the colonies appear homogeneous.

Litmus glucose agar. Rather thin growth with pellucid areas, dry, with no tendency to spread. Acidity develops in twenty-four hours and increases.

Litmus glucose agar colonies. Thin transparent moist surface colonies and punctiform deep colonies. Under low power homogeneous with regular edges. Slight acidity produced.

Gelatin. Funnel-shaped liquefaction rather rapid. No scum formation.

Gelatin colonies. Irregular and round dirty-white colonies, homogeneous with circumscribed edges. Rapid liquefaction.

Broth. Turbidity with heavy flocculent precipitate. No scum.

Peptone. Similar to growth in broth but less luxuriant. No scum.

Potato. Raised granular cream-colored abundant growth.

Litmus milk. Slow development of acid and later coagulation. Partial digestion of the casein.

Blood serum. Heavy moist yellow-brown growth. Depression of the growth with slow liquefaction of the serum.

Fermentation tubes. Glucose: Turbidity and faint granular precipitate in bowl and cloudiness in closed arm. No pellicle. Reaction faintly acid.

Sucrose: Turbidity in bowl and neck of tube. No pellicle. Reaction alkaline.

Lactose: Turbidity in bowl and neck. No pellicle. Closed arm cloudy. Reaction acid.

Thermal death point. Spores survive 5 pounds in the autoclave but are destroyed by 10 pounds pressure. They are killed by thirty minutes exposure to steam in the Arnold sterilizer.

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LAUBACH AND RICE 1916 J. Bact., 1: 513.
LAWRENCE AND FORD 1916 J. Bact., 1: 273.

The drawings were made by Mr. W. P. Didusch from smears stained with gentian violet with a Bausch and Lomb microscope No. 12 \times eye piece and one-twelfthth fluorite oil-immersion objective giving a magnification of 1260 diameters.

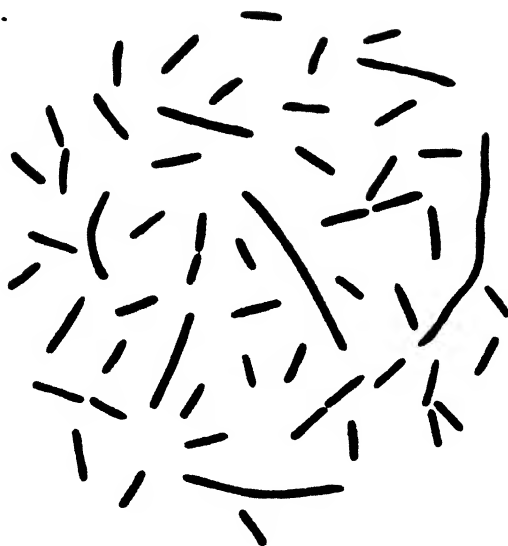


FIG. 1. *BACILLUS BADIUS*. PLAIN AGAR, TWENTY-FOUR HOURS

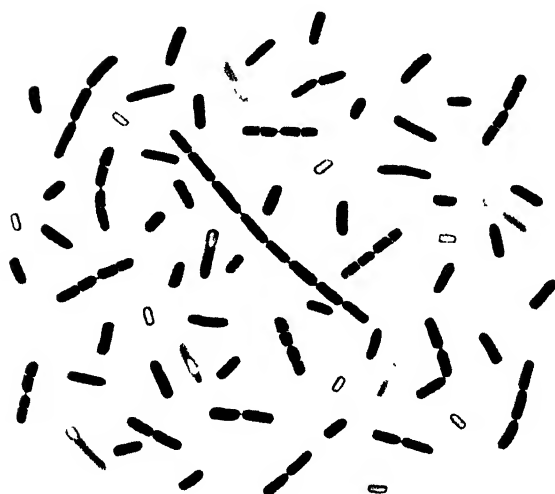


FIG. 2. *BACILLUS BADIUS*. GLUCOSE AGAR, TWENTY-FOUR HOURS

(Batchelor: Aerobic Spore-Bearing Bacteria in Children)



FIG. 3. *BACILLUS BADIUS*. PLAIN AGAR. OLD CULTURE

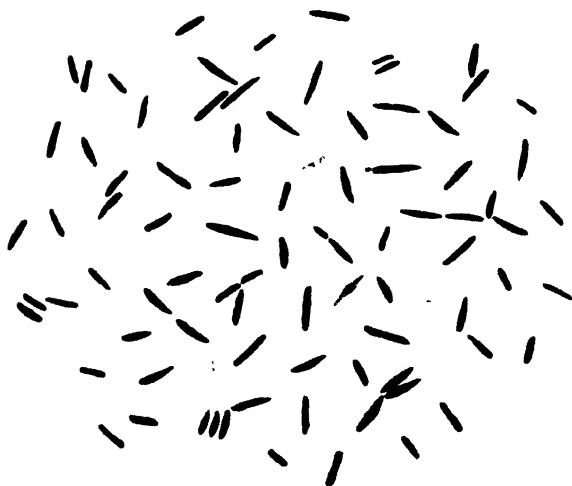


FIG. 4. *BACILLUS FUSUS*. PLAIN AGAR, TWENTY-FOUR HOURS
(Batchelor: Aerobic Spore-Bearing Bacteria in Children)

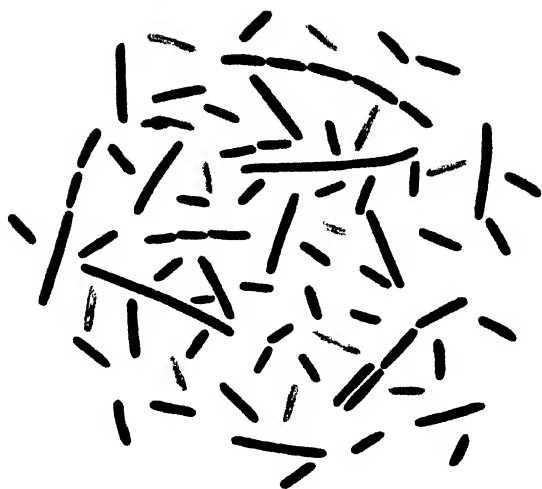


FIG. 5. *BACILLUS FUSUS*. GLUCOSE AGAR, TWENTY-FOUR HOURS

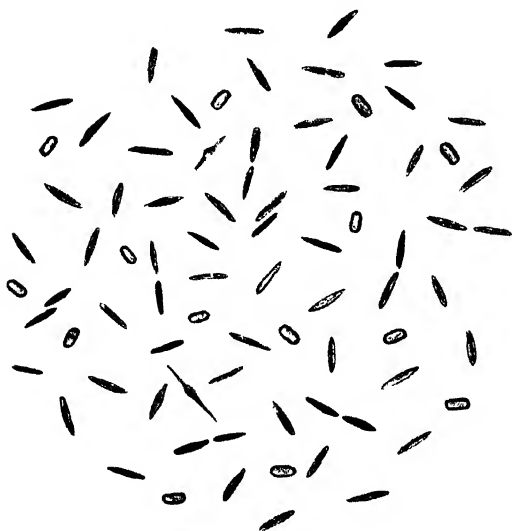


FIG. 6. *BACILLUS FUSUS*. PLAIN AGAR, OLD CULTURE

(Batchelor: Aerobic Spore-Bearing Bacteria in Children)

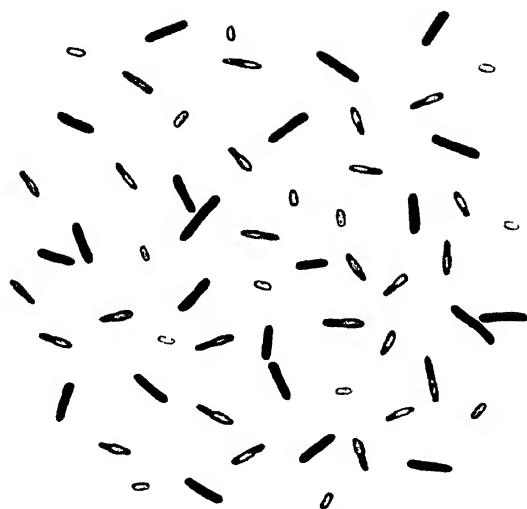


FIG. 7. *BACILLUS TRITUS* PLAIN AGAR, FORTY-EIGHT HOURS

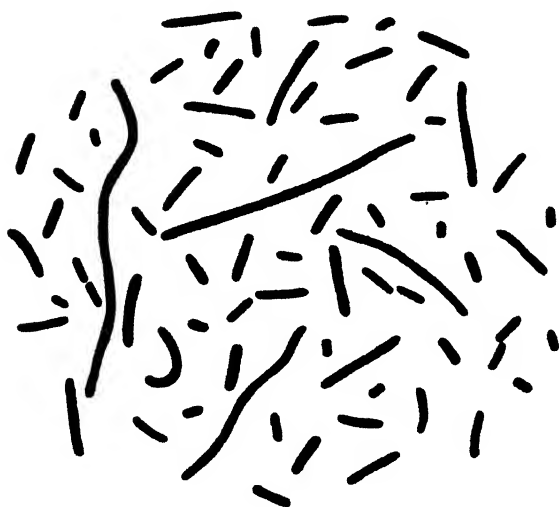


FIG. 8. *BACILLUS TRITUS* GLUCOSE AGAR, FORTY-EIGHT HOURS

(Batchelor: Aerobic Spore-Bearing Bacteria in Children)

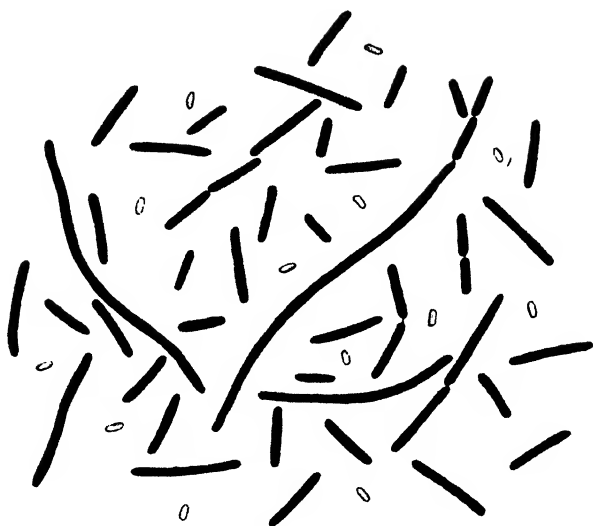


FIG. 9. *BACILLUS TRITUS*. PLAIN AGAR, FOUR DAYS.

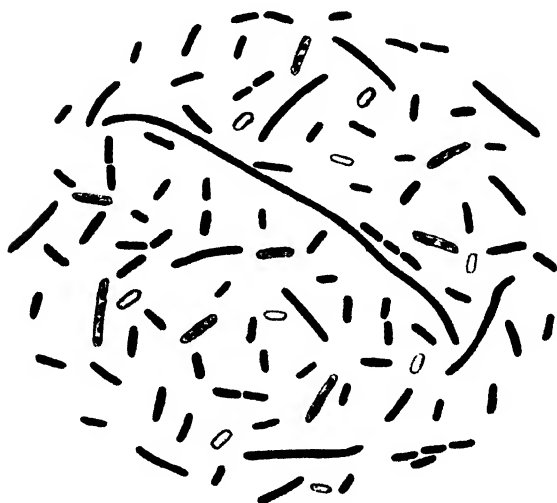


FIG. 10. *BACILLUS LAUTUS*. PLAIN AGAR, FORTY-EIGHT HOURS.
(Batchelor: Aerobic Spore-Bearing Bacteria in Children)



FIG. 11. *BACILLUS LACTUS*.—GLUCOSE AGAR, FOUR DAYS.

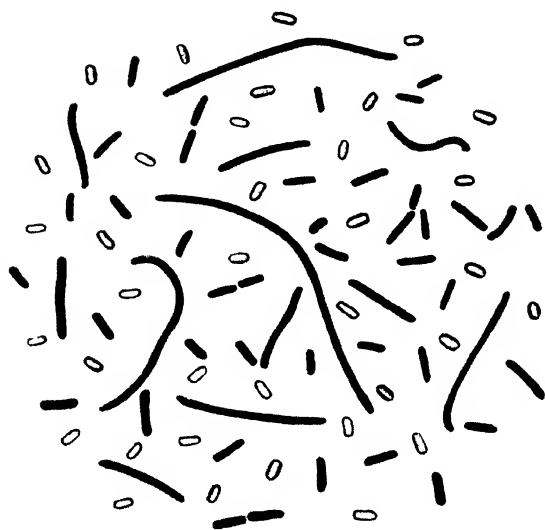


FIG. 12. *BACILLUS LACTUS*.—PLAIN AGAR, FOUR DAYS.

(Batchelor—Aerobic Spore-Bearing Bacteria in Children)



FIG. 13. *BACILLUS FLEXUS*. PLAIN AGAR, SIX HOURS

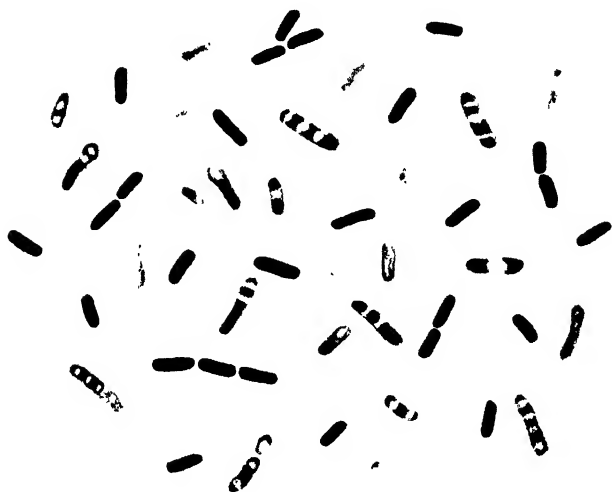


FIG. 14. *BACILLUS FLEXUS*. GLUCOSE AGAR, TWENTY-FOUR HOURS

(Batchelor: Aerobic Spore-Bearing Bacteria in Children)



FIG. 15. *BACILLUS FLEXUS*. PLAIN AGAR. OLD CULTURE.

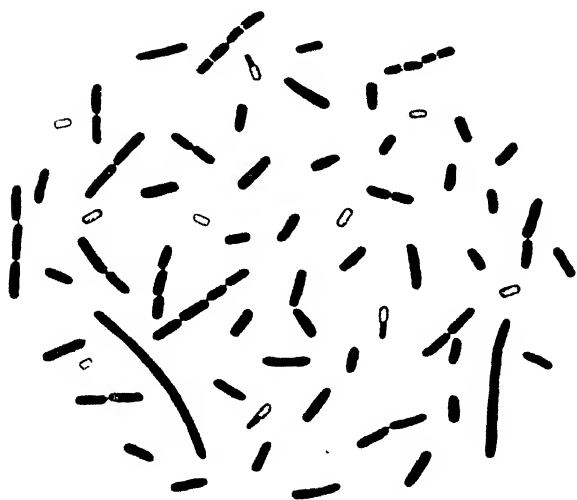


FIG. 16. *BACILLUS FLEXUS*. PLAIN AGAR, OLD CULTURE

(Batchelor: Aerobic Spore-Bearing Bacteria in Children)

NON-LACTOSE FERMENTING BACTERIA FROM POLLUTED WELLS AND SUB-SOIL¹

I. J. KLIGLER

*From the Laboratories of the Rockefeller Institute for Medical Research,
New York*

Received for publication February 1, 1918

While *B. coli* is generally accepted as a satisfactory index of pollution, it is always desirable to detect the presence of specific pathogens in any suspected material. The methods for isolating such pathogens, though highly developed, are far from satisfactory. In water, one encounters the problem of high dilution, while in contaminated soil, the presence of large numbers of organisms of the *B. proteus* group interferes greatly with the successful detection of typhoid, dysentery, and related types of bacilli. The failure to find the latter organisms does not, therefore, imply their absence, though it may indicate that they are not present in large numbers.

METHODS

The samples of soil and water were collected in sterile wide mouth, glass stoppered bottles. The soil specimens were removed to sterile Petri dishes, thoroughly mixed, and portions of 10 to 25 grams weighed into sterile glass stoppered bottles. Sterile water was then added in the proportion of 2 cc. of water to 1 gram of soil, the bottles shaken vigorously for a few minutes and then allowed to stand until the heavy particles had settled to the bottom. The supernatant suspension was then tested by two or all of the following methods:

¹ Work conducted under a grant of the International Health Board of the Rockefeller Foundation, New York.

Direct plate method. The water and soil suspensions were plated direct on Endo and brilliant green plates, 0.1 cc. being spread on the former, and 0.2 cc. on the latter, respectively. The plates were incubated for twenty-four hours and suspicious colonies fished to Russell double sugar tubes. All the Gram negative non-lactose fermenting bacilli were kept for further study.

Bile enrichment method. Portions of 10, 2, and 0.2 cc. of the soil suspension and 10, 1.0, and 0.1 cc. of the water were inoculated into lactose bile and the tubes incubated for forty-eight to seventy-two hours. A loopful of fluid from all the tubes that showed gas was then spread on both brilliant green and Endo plates, and the suspicious colonies appearing after incubation for twenty-four hours were inoculated into Russell double sugar tubes, and treated as above.

Agglutination with polyvalent anti-coli serum. This method was the converse of that used by Adami and Chopin (1904). Adami and Chopin reported successful results in the isolation of *B. typhi* from water with the aid of antityphoid agglutinating serum. Preliminary tests of this procedure gave unsatisfactory results, because *B. coli* invariably agglutinated together with the *B. typhi* in sufficiently large numbers to crowd out the latter on plates. It seemed that a polyvalent colon agglutinating serum would be more generally applicable, in that it would remove the *B. coli* and leave the specific pathogens, as well as the other non-lactose fermenting bacilli, in the supernatant fluid.

By inoculating 5 different varieties of *B. coli* into a sheep, a serum was obtained that could agglutinate 50 to 60 per cent of a heterogeneous collection of 50 strains of *B. coli*. No agglutinating serum could be obtained against *B. aerogenes* and a type of *B. coli-communior*, which is apparently very close to the capsulated *B. aerogenes*.

This anticolon agglutinating serum was used successfully in the isolation of non-lactose fermenting bacilli from a few samples of soil, water and feces. The procedure was as follows: The serum was added to beef bouillon or peptone broth in a concen-

tration of 1:100. Then 10 cc. of water or the soil suspension or a loop of feces were added to 10 cc. of broth, and the tubes incubated until flocculation was visible. (The period of incubation depended, of course, on the number of *B. coli* present at the time of incubation and varied from two to six hours). The tubes were then centrifugalized at low speed for 5 minutes, and the supernatant fluid plated on Endo and brilliant green plates. Two samples of soil, 2 of water, and 2 stools from patients suffering with summer diarrhea yielded successful results with this method. From the stools practically pure cultures of *B. dysenteriae* (Flexner) and the Morgan bacillus respectively were obtained with this procedure.

RESULTS

By the use of the methods outlined above, either singly or in combination, non-lactose fermenting, non-liquefying bacteria were obtained from 15 polluted wells and 14 contaminated subsoil samples. In but one instance were members of this group obtained from both the well and soil of the same premises, but even in this case the organisms possessed different biological and serological characters.

The predominant organism obtained from the wells differed from the one found in the sub-soil. The fifteen wells yielded 2 organisms resembling the acid type of *B. dysenteriae*, 2 strains of the Morgan bacillus no. 1, 9 para-enteritidis, and 2 paracolon bacilli. The soil samples, on the other hand, gave 8 dysentery-like bacillary strains, 7 of which corresponded culturally with the acid and 1 with the Shiga type; 4 para-enteritidis, and 2 paracolon bacillary strains. In only two instances were non-lactose fermenting bacilli obtained from sub-soil at a distance greater than 2 feet from a privy pit. Both were sandy soils examined during the wet period. The distance from the privy in these two cases was 10 feet and the depths were $7\frac{1}{2}$ to $8\frac{1}{2}$ feet respectively.

It is noteworthy that the prevalent type in wells belongs to the paratyphoid or para-enteritidis bacterial group, while that

TABLE 1

The fermentation and agglutination reactions of the non-lactose fermenting bacilli isolated from polluted wells and soil

NUMBER	SOURCE	FERMENTATION REACTION							AGGLUTINATING SERA				TYPE RESEMBLED
		Glucose	Mannite	Sucrose	Xylose	Arabinose	Rhamnose	Salicin	Enteritidis	Paratyphi B	Hog cholera	Dysentery	
155	Soil	+	+	-	-	+	-	-	-	-	-	-P	Flexner dysentery
493	Soil	+	+	-	-	+	-	-	-	-	-	+(100)F	Flexner dysentery
395	Well	+	+	-	-	+	-	-	-	-	-	+(100)F	Flexner dysentery
112	Soil	+	+	-	-	-	-	-	-	-	-	-P	
384	Soil	+	+	-	-	-	-	-	-	-	-	-P	
1552	Soil	+	-	-	-	-	-	-	-	-	-	-P	Shiga (not toxic)
75	Soil	+	+	+	+	+	-	-	-	-	-	±(500)F	Strong dysentery
84	Soil	+	+	+	+	+	-	-	-	-	-	+(100)F	Strong dysentery
512	Soil	+	+	+	+	+	-	-	-	-	-	+(1000)P	Strong dysentery
437	Well	+	+	+	+	-	-	-	-	-	-	-P	?
388	Well	+	+	+	+	-	+	-	-	-	-	±(1000)P	?
60	Soil	+	+	+	+	+	+	-	-	-	-	±(500)F	?
57b	Well	*	-	-	-	-	-	+	+1000	-	-	-	Morgan no. 1
318	Well	*	-	-	-	-	-	+	+1000	-	-	-	Morgan no. 1
286	Well	*	*	-	*	*	*	-	++100	-	-	-	Enteritidis
30	Well	*	*	-	*	*	*	-	±100	-	-	-	Enteritidis
33	Well	*	*	-	*	*	*	-	-	-	-	-	Enteritidis
46	Well	*	*	-	*	*	*	-	+100	±100	-	-	Enteritidis
57a	Well	*	*	-	*	*	*	-	±100	±500	-	-	Enteritidis
91	Soil	*	*	-	*	*	*	-	±500	-100	-	-	Enteritidis
143	Soil	*	*	-	*	*	*	-	±500	±100	-	-	Enteritidis
206	Well	*	*	-	*	*	*	-	±100	+100	-	++(500)P	Enteritidis
213	Soil	*	*	-	*	*	*	-	-	-	-	-	Enteritidis
227	Well	*	*	-	*	*	*	-	+100	-	-	-	Enteritidis
294	Soil	*	*	-	*	*	*	-	+100	-	-	±100	Enteritidis
410	Well	*	*	-	*	*	*	-	±500	+100	-	-	Enteritidis
417	Well	*	*	-	*	*	*	-	+1000	-	-	-	Enteritidis
507	Soil	*	*	-	*	*	*	+	-	±500	++100	-	Paracoli
461	Soil	*	*	-	*	*	*	+	-	+100	-	-	Paracoli
424	Well	*	*	-	*	*	*	+	-	±500	-	+ colon ser. 500	Paracoli
50	Well	*	*	-	*	*	*	+	-	-	-	-	Paracoli

Summary of Table I

Total number of specimens.....	29
Number of wells.....	15
Number of soils.....	14

TYPES FOUND IN	DYSENTERY-LIKE BACILLI	PARA-ENTERITIDIS BACILLI	PARACOLON BACILLI	MORGAN BACILLI
Wells.....	2	9	2	2
Soil.....	8	4	2	0

+ = acid without gas.

* = acid with gas.

Note. The salicin positive, indol positive strains are considered as paracolon; the indol negative strains are classed as para-enteritidis. The bacilli fermenting acid without gas are for convenience thrown together into a "dysentery-like" group.

P = polyvalent antidyentery serum.

F = Specific Flexner antidyentery serum.

found in polluted soil resembles the dysentery bacillus. Smith and Moore (1893) found members of the paratyphoid group in the feces of normal cattle, swine, and other domestic animals. Savage (1906-1910) carried out an extensive investigation on the prevalence of non-lactose fermenting bacilli in excreta of man and animals. He reports the presence of para-Gärtner bacilli in large numbers in normal swine, calves, horses, and mice; but claims that in man they are encountered only infrequently and in small numbers. The true Gärtner bacillus was detected by him once only (in human excreta); the others were what he terms the para-Gärtner bacilli, which differed from the type organism in their behavior toward dulcitate and salicin and in their agglutination reactions.

The paratyphoid-like organisms isolated from both wells and soil all belong to the para-enteritidis group. Their reactions, source, etc. are shown in the table. They differ from the *B. paratyphi A* in their power to ferment xylose; from the *B. paratyphi B* and *B. enteritidis* in their failure to attack dulcitate (with one exception); and from the hog cholera bacillus in their ability to break down arabinose. They also fail to agglutinate in the higher dilutions with *B. paratyphi B*, *B. enteritidis*, or hog cholera sera. Only one strain (294) presents the characteristic

reactions of the *B. enteritidis*, but even this strain is not agglutinated by the serum prepared from a typical strain in a dilution higher than 1:100; although the titre of the serum was well over 2000. All the strains may therefore be considered members of the para-enteritidis group frequently present in excreta of domestic animals, particularly swine (Savage 1906-1910).

The dysentery-like organisms, or, to be more exact, the non-gas producing bacilli, were the forms most frequently isolated from soil and least frequently from wells. None of the strains was true to the dysentery type with respect both to fermentation and agglutination. Two strains were partially agglutinated in a 1:1000 dilution of a polyvalent serum, and two others in 1:500 of either polyvalent or specific Flexner serum. These agglutinated cultures all fermented sucrose and xylose and resembled, therefore, the Strong type. Three strains were agglutinated only in dilution of 1:100 of the polyvalent or specific Flexner serum, although two of them were culturally identical with the Flexner bacillus. The other strains were not agglutinated at all. It is probable that these bacilli bear the same relation to the true dysentery organisms, that the para-Gärtner bacilli do to the *B. enteritidis*. Organisms of this type have been found by Lewis, (1911-1913) Alexander, (1913) and others in stools of normal children and those suffering from diarrhea. As far as I am aware, they have not been reported from other sources; nor have they been shown to be definitely associated with any pathological conditions, although they are reported to be more frequently found in the excreta of children with diarrhea than in normal stools.

Paracolon bacilli are often found in both human and animal excreta. The usual strains ferment salicin and may or may not ferment dulcitol. They also produce indol (Savage, 1906-1910) (Kligler, 1914). Their presence is of less significance than that of the other non-lactose fermenting bacilli, since they are as ubiquitous as the typical colon bacilli.

The Morgan bacilli are of interest because they have been definitely associated by the discoverer with the summer diarrheal diseases of children. Organisms of this type were isolated from

two wells but not from the soil. Morgan and Ledingham, (1909) Lewis, (1911-1913) Alexander, (1913) and others report the frequent presence of this bacillus in normal children and its more regular occurrence in children suffering from diarrhea. Morgan and Ledingham also found the bacillus in one cow out of 18 examined but not in the horse; while Lewis isolated it from 5 out of 20 mice. Whether it occurs in other animals is not known.

While it is hard to detect any absolute relations between the non-lactose fermenting bacilli and the sources of soil and water pollution, the fact does seem significant that the organisms most often found in wells are those more frequently encountered in the excreta of domestic animals, while the predominant sub-soil bacteria are more characteristic of human excreta. This relationship between type and host is not definitely established, but is rendered highly probable by the results recorded in the literature. If the indication proves to be a fact, the results reported above suggest a surface origin of the well pollution, and a human origin of the sub-soil contamination, (the adjacent pits were indeed obviously responsible for the latter). A study of the non-lactose fermenting bacilli usually found in the intestines of domestic animals and human beings and their resistance to storage in soil and water might prove of decided value in tracing the source of pollution.

SUMMARY

A study of the biological and serological characters of the non-lactose fermenting bacilli isolated from polluted well waters and contaminated sub-soils is reported. It appears that the type of bacillus predominant in polluted well waters differed from that isolated from polluted sub-soils. The former belonged to the gas producing para-enteritidis group most common in the intestinal tract of swine, cattle, and other domestic animals. The latter, on the other hand, was a non-gas producing form resembling the bacillus found by Morgan, Lewis, and others in the stools of normal individuals, as well as in those of people suffering from diarrhoea.

This relationship between the type of the non-lactose fermenting bacilli and host, though not definitely established, is suggestive. It is possible that a careful study of the non-lactose fermenting bacilli commonly found in the intestinal tracts of man and domestic animals would furnish the basis for an index of the source of pollution.

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ON NITRIFICATION

II. INTENSIVE NITRITE FORMATION IN SOLUTION

AUGUSTO BONAZZI

Contribution from the Laboratory of Soil Biology, Ohio Agricultural Experiment Station, Wooster, Ohio

Received for publication, February 25, 1918

INTRODUCTION

In the first paper from this laboratory dealing with nitrification, (1915) the importance of the problem and the difficulties in its study were discussed. The very great need of pure culture studies on an appropriate synthetic medium was pointed out; but we had not at that time succeeded in consistently producing really vigorously growing cultures of either the nitrite or of the nitrate former. The use of soil extracts in the preparation of the media, which procedure now finds quite extended use in soil bacteriological technique, gave us practically no encouragement, and this finding has been confirmed by subsequent work. Somewhat better growths were obtained on porous media, and among those tried ignited soil seemed to possess the greatest promise. It has some advantages over ordinary soil or mixtures of quartz sand and humus, yet it is not a synthetic substance of known composition and properties, and does not permit us, therefore, to determine with certainty the cause or causes of its beneficial action. Our quest for the conditions or set of conditions which would with certainty give rise to vigorous growths on media of known composition was not abandoned, and after long continued and persistent effort we have been able to stimulate with satisfying certainty luxuriant growths of that one of these forms, the nitrite producer, which was chosen for exhaustive study.

HISTORICAL

The slow and uncertain growth of the organisms of nitrification has been recognized by practically every one of the host of investigators who have studied the process. The reason why a process which takes place so widely, and often so vigorously, in nature is so slow in synthetic laboratory cultures has been the subject of a considerable amount of discussion among modern investigators, but unfortunately little attention has been given to the actual study of the question.

To attempt to review all the literature on methods of cultivating and growing the nitrifying organisms is beyond the scope of this paper. The uncertainty attending the growing of these organisms on synthetic media has led numerous writers to use ordinary soil as a medium, contending that it approaches the "natural" conditions in the field. Because of the one redeeming feature that this medium possessed, that of producing a relatively rapid growth of the organisms, all its undesirable characteristics, such as unknown and variable composition, difficulty of manipulation, and general inadequacy for bacteriological procedures, were overlooked. As an example of extreme views on the comparison of soils and solutions as media for the study of nitrification may be cited again the work of Stevens and Withers (1909) who believed that the physiological processes were more or less fundamentally different in soils from those in solutions, and that the organisms adapted to producing rapid action in solutions, e.g., in sewage effluents, were not identical with those of soil. The fallacy of this type of reasoning has been pointed out and the general superiority of solutions over soil has been emphasized by Löhnis and Green (1914).

In reading the literature on the subject of nitrification one is impressed with the fact that the earlier workers were more aware of the importance of aeration to the process than have been the modern workers. Thus Schlösing and Müntz (1877) found that by passing a current of sterile air through sewage containing calcium carbonate the ammonia contained in the sewage was transformed to nitrate. Thus, we see, that at this

early time the basis was laid for the development of the activated-sludge method of sewage purification. The air bubbling through the sewage was capable of activating a rapid nitrification without adding to the medium any living bacterial cell. These same authors (1879a) pointed out later that aeration was not only useful but essential to nitrification, and that it may be obtained either by bubbling filtered air through a liquid medium or by spreading this medium in very thin layers. Moreover, they emphasized again (1879b) the influence of aeration on the process of nitrification and in a still later paper (1889), Schlösing showed by gas analysis that oxygen is actually taken up during the process of nitrification, and that the quantity of oxygen so consumed bears a constant ratio to the amount of nitrogen nitrified. It is of further interest in this connection to note that Winogradsky (1890a) started his cultures in "flasks with flat bottom of an exceptionally large diameter, in which the liquid formed a shallow layer" and that a little later (1890b), in criticising his own work, he says of these same cultures: "my flasks with a small volume of liquid imperfectly aerated could not be so favorable to the phenomenon of oxidation."

The remarkable work of Boullanger and Massol (1903, 1904) points out again how essential it is in nitrification experiments to obtain good aeration. By the addition of scoria to the solution, and gentle movement at regular intervals so as to renew the film of solution wetting the scoria an increase in the velocity of nitrification was observed. That this hastened nitrification may also be due to some cause other than simple aeration should be considered possible in the light of the recent work bearing on the beneficial action of continued agitation of the liquid cultures of *B. tuberculosis*.

Of special interest and importance in this connection is the work of Lucet (1913) who found that slow and continuous agitation was not only favorable to aerobes but to anaerobes likewise: thus cultures of the organisms of anthrax, cholera, typhoid, diphtheria, glanders, dysentery, tetanus, pseudotuberculosis, leprosy, symptomatic anthrax, erysipelas and cultures of *B. pyocyaneus* were benefited by such treatment.

The explanation of this peculiar phenomenon is sought by Lucet in (1) a better utilization of the medium by a continued contact of new utilizable food with the cells, and (2) a better distribution of the products of cellular activity, which would otherwise accumulate in the immediate vicinity of the cell and would only slowly diffuse.

The intensive nitrification obtained by Müntz and Lainé in recent years, by allowing ammoniacal solutions to flow through peat and carbon black inoculated with nitrifying organisms (1906a, 1906b) should probably be explained both by a greater aeration and an action similar to the one mentioned by Lucet; the same may be said of the work on intermittent and continuous filters by Roland and Gallemand and by Miss Chick.

The effect of the ratio of diameter to depth established by Löhnis (1905) and by Löhnis and Green (1914) is typical of the results to be obtained when the effect of aeration is segregated from the effect of the renewal of environment; unfortunately the importance of the results obtained by these authors is limited by the indefinite significance of such a ratio. Lately Barthel (1910), forgetful of the fact that the practice which he followed had been before recommended by Schlösing and Müntz (see above), published the details of a method for the activation of nitrification by passing a current of air through a relatively high column of solution.

From these quotations, it is evident that the benefit to be derived by the aeration of nitrifying cultures is something which requires no further proof; but while the solution cultures have been discarded by some investigators as unfit, and other media have been suggested to replace them, it is plain that aerated solution cultures should be given a thorough trial.

As mentioned above, and as pointed out by Löhnis and Green, the chief reason why the solution cultures have been discarded by modern investigators, lies in the fact that the workers who discarded them did so after using them under such conditions as would naturally make them unfit for any aerobic culture. In some cases as much as 300 cc. of solutions were used in 1000-cc. Erlenmeyer flasks which arrangement gave a depth of the layer

of solution of several centimeters at complete rest during the whole period of incubation. From the work of Schlösing and Müntz and of Barthel, it would seem that the depth of the solution might be disregarded only if other means are provided for the aeration of the solution.

PHYSIOLOGICAL STUDY OF THE CULTURES

In all our early work care was taken to observe the same precautions which were observed by the early investigators. Very thin layers of solution were used, so as to permit as thorough an aeration as was possible; usually 25-cc. portions of solution were used in 250- or 300-cc. Erlenmeyer flasks, the layers so obtained measuring 3 or 5 mm. in depth.

The standard medium used was that recommended by Omelianski (1899) for the isolation of the nitrite forming organism, and possessed the following composition:

Ammonium sulphate	2.0 grams
Sodium chloride	2 0 grams
Potassium phosphate	1 0 grams
Magnesium sulphate	0.5 grams
Ferrous sulphate	0 4 grams
Water	1000 cc.

sterile magnesium carbonate was added to each culture flask after sterilization. The potassium phosphate used was dibasic.

Nitrite production in this medium under the conditions stated above was slow and unsatisfactory. Indeed in many cases the process failed to take an active course, in some cases failing altogether, in others declining slowly after a period of feeble growth. With cultures of relatively strong action the following behavior was observed.

On October 6, 1916, 25 cc. of the ordinary Omelianski solution in 250-cc. Erlenmeyer flask, which received after sterilization the addition of basic magnesium carbonate, was inoculated with 1 cc. of a pure mother culture.¹

¹ The term pure culture is used in all our work to denote a culture that when inoculated in bouillon of either *acid* or *alkaline* reaction gives no growth after 10 days and that gives a uniform picture on microscopical observation.

On October 27, after twenty-one days incubation at 30°C. a sister culture was tested and found still to contain some ammonia. On November 1, 1916, after twenty-six days incubation at 30°C., the culture was analyzed and found to contain 8.03 mgm. of nitrogen as nitrite. The original 25 cc. of solution contained 10.6 mgm. nitrogen as ammonia.

Another culture, sister of the one first mentioned, when tested on November 21 was found to contain considerable quantities of ammonia, and when analyzed after twenty-six days incubation at 30°C. was found to contain 7.94 mgm. nitrogen as nitrite.

From what has been stated, it is evident that the action of the cultures was a relatively slow one. It should be noted here that the flasks remained at perfect rest until tested, and then again until analyzed.

Modifications of the original Omelianski solution were unsuccessfully tried. The first attempts were made with the organisms of nitrate formation, using soil extracts in place of water in the preparation of the nutritive solution. No encouraging results being obtained in this direction, attempts were made to modify the solution by the addition of compounds of definite composition. Omelianski solutions for nitrite formation were prepared to which were added small amounts of potassium nitrate, zinc sulphate, potassium iodide, potassium bromide, ammonium-potassium sulphate; as a further precaution, the quantity of ammonium sulphate was reduced to one-half the quantity recommended by Omelianski.

As can be seen in the accompanying table very little if any benefit at all was experienced by such modification. An average of four cultures for each treatment of a nearly pure nitrosomonas gave the results summarized in table 1.

TABLE 1

TREATMENT	MGM. OF NITRITE IN 25 CC. OF CULTURE, AFTER THIRTEEN DAYS
Ordinary Omelianski solution	3.08
Modified solution.....	4.51

The use of solutions made so as to contain the iron-aluminium-silico-phosphate, recommended by Kaserer (1911) as adapted to *Azotobacter* organisms, gave no more encouraging results than the trials reported above.

The addition of dialized silicic acid to the solutions was likewise of no avail.

Failing to obtain the solution of our difficulties by this method of attack attention was directed to the question of oxygen requirements of the "*nitrosomonas*."

Trials were then made in the use of very large surfaces, and to that end the flat bottomed Fernbach antitoxin flask was adopted. This flask has been used by Barthel in his work on *Azotobacter* and found satisfactory.

Encouraging results were immediately obtained by the use of these receptacles: certain cultures utilized in four days a dose of 5 cc. of a 5 per cent ammonium sulphate solution, as determined by the disappearance of the ammonia from the nitrifying solutions, the control flasks retaining all or nearly all their ammonia during the same period.

It was then thought that the use of some solid material to be periodically wetted by the solution would enhance the process of oxidation. This is essentially what Boullanger and Massol did, and we therefore searched for the nature and composition of the "scoria" recommended by them. The following paragraph giving a description of the material in question is quoted from a personal communication received from Professor Boullanger: ". . . the scoria that we have used are simple residues of the combustion of coal in the grates of generators (black, agglomerated and uncombustible residue)"²

Since this communication was received after the work in this direction has been initiated, we were unable to repeat exactly Boullanger and Massol's experiments, and ignited soil was used instead of "scoria."

² Les scoria que nous avons employées sont de simples résidues de la combustion du charbon sous les grilles des générateurs (parties agglomérées, noires, et non combustibles).

Some soil from Paulding County, Ohio, was sifted so as to separate it in its various sizes, taking care not to crush the clumps and agglomerates. These clumps were separated from the finer material and then ignited at a temperature of 700°C.

Three hundred grams of this ignited soil were placed in a Fernbach flask together with 100 cc. of Omelianski's solution and basic magnesium carbonate: after inoculation the flask was inclined on one side so that the solution, after wetting all the soil clumps, collected at the lower part of the flask. This inclination was changed twice daily so that a fresh supply of food came in contact with the bacteria lodging in the soil clumps, at each such period. The result of this trial was quite encouraging. A rapid nitrification ensued, so that for over a week it was necessary to add daily a dose of food corresponding to 1 cc. of 10 per cent sterile $(\text{NH}_4)_2\text{SO}_4$ solution.

Repetition of this experiment led to the same results. Unfortunately, due to the preliminary nature of these experiments, no quantitative data were collected and it is therefore impossible to state whether all the ammoniacal nitrogen was nitrified or whether a considerable portion of it was volatilized from the alkaline medium. Nevertheless, the results obtained were such as to warrant a study of aeration and a new less complicated line of attack was adopted.

The benefits to be derived from the shaking or agitation of a culture, as indicated by the work of Lucet and of Boullanger and Massol, were used as guiding principles. The enhanced nitrification realized by the last mentioned investigators when they adopted the means of aeration used in the Luxemburg method of vinegar production as well as the results obtained in this laboratory in large Fernbach flasks were kept in mind.

In attempting to obtain strong aeration of our cultures we aimed at an apparatus which would unite the ample contact with oxygen obtained in the German method of vinegar production, with a constant container such as is used in the Luxemburg method of vinegar production. A klinostat on which large Fernbach flasks were placed served the purpose perfectly.

The flat bottoms of the flasks had a surface of approximately 300 sq. cm. and one hundred cc. of solution in these flasks in a horizontal position formed a layer 0.7 cm. deep. The klinostat having an inclination of $\pm 5^\circ$, the flasks were in such a position that the solution would flow to the lower side leaving a layer of ± 1 mm. on the upper half of the flask: i.e., over a surface of ± 150 sq. cm.

The planes of the klinostat performed one revolution every three to five minutes so that this very thin layer was continually shifting to a new surface, and only returned to its original position once every three to five minutes.

By means of this rotary movement, a very slow centrifugal action and a constant mixing of the solution was accomplished so that it was not the same portion of solution which returned on a definite surface at each turn of the klinostat. The accompanying sketch will serve to elucidate the description.

In this work the determination of the transformed nitrogen was accomplished by finding the increase of nitrite nitrogen in the culture after definite periods of time. In every case 5 cc. samples were taken from the cultures, with sterile pipettes, diluted to definite volume in volumetric flasks, and sub-sampled for duplicate analysis. Aliquots of 20 or 25 cc. of this diluted material were analyzed by the starch-iodide method developed in this laboratory (Davisson, 1916) using 0.02 N or 0.01 N. thiosulphate solution.

In order to avoid any personal error in the analytical technique, several samples were analyzed by two persons. Although the absolute results obtained by the two persons varied slightly, the relative differences due to the action of the organisms in the cultures were found to be the same in each case.

The flasks were weighed each time before sampling and the volume of the solution contained on each date determined by subtracting from this weight the tare of the empty flask. The cotton plugs were covered by beakers to reduce to a minimum the possibility of contamination.

The first culture started by this method was inoculated with a nitrite forming organism which had been cultivated in this

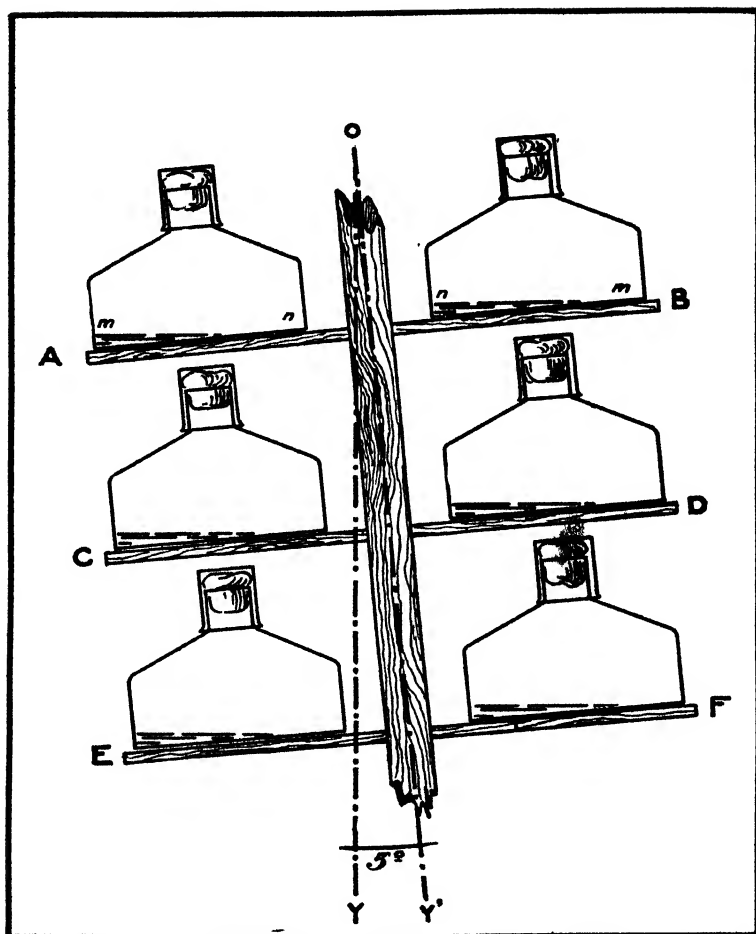


FIG. 1. The klinostat in performing one-half revolution on the axis OY' , which is removed from the vertical axis OY by 5° , turns the planes AB , CD , EF , to the opposite position so that the points A , C , E , on the periphery of the planes come to occupy the positions B , D , and F , respectively. The solution then flows from m to n in the flasks on the left side of the figure, and from n to m in the flasks on the right side of the figure. When the klinostat has rotated over an angle of 360° , the solutions assume again the position pictured in the figure.

laboratory for the last three years, and had not been grown in soil for that same period of time.

The results of our first experiment on intensive nitrite formation are shown in table 2. The results are expressed as milligrams of nitrogen oxidized to nitrite per day in 100 cc. of culture.

Leaving aside, for the present, the irregular action of the culture, the rate of oxidation must be considered to be a very rapid one. It surpasses manyfold the action of the ordinary culture which one is accustomed to study and indeed the action of this same organism under the ordinary laboratory conditions. In the light of the above results, many more cultures were started and all gave equally satisfactory results.

TABLE 2
Results on intensive nitrite formation

PERIOD OF INCUBATION	DAILY PRODUCTION OF NITRITE N
First three days of incubation.. . . .	12 69
Next two days of incubation.	10.83
Next one day of incubation...	12.43
Next two days of incubation	9 38
Next two days of incubation	14.48
Next three days of incubation.. . . .	17.70
Next one day of incubation	7 30
Total in fourteen days of incubation	168 92

Table 3 summarizes the action of two other cultures which were started under the same conditions, the results being expressed as before as milligrams of nitrogen oxidized to nitrite per day per 100 cc. of culture solutions.

Here, the weak action during the first four days is due to the small inoculum used and the relatively long period of incubation which caused the amount of nitrogen oxidized during the fourth day to be distributed evenly over a period four times its length.

Leaving aside the irregularities which are due to special treatments, the strong rate of oxidation stands out very prominently. As has already been mentioned, similar results were obtained in every case in which cultures in Fernbach flasks were rotated on a klinostat.

In order to furnish further proof of the possibilities of nitrification when suitable conditions are provided, the results obtained from another series of cultures are summarized in table 4 and graphically shown in figure 2. This set of cultures which was started in order to study the regularity of the phenomenon of nitrite formation under various concentrations of ammoniacal salts, gave very satisfactory results. The results

TABLE 3

PERIOD OF INCUBATION	DAILY PRODUCTION OF NITRITE N	
	Culture I	Culture II
First four days of incubation.....	0.77	0 94
Next three days of incubation	9.69	7.61
Next two days of incubation.....	8.31	14 17
Next two days of incubation.	9 86	10 15
Next two days of incubation	13.28	18 13
Next four days of incubation	9.86	9 75
Total in seventeen days of incubation.. . . .	173.93	194 49

TABLE 4

Daily production of nitrite nitrogen (expressed as milligrams of nitrogen per 100 cc. per day)

NUMBER OF CULTURE	DAILY RATE											TOTAL IN 28 DAYS OF INCUBATION
	First 7 days	Next 2 days	Next 3 days	Next 1 day	Next 3 days	Next 1 day	Next 2 days	Next 3 days	Next 1 day	Next 1 day	Next 4 days	
2	2.09	7 90	6.65	12 00	3 96	4 75	12 75	6.47	11.84	9 17	8 09	187 38
3	2 00	9.51	6 45	7 97	4 67	14.74	8 60	7.34	9.75	9.45	11 46	199 05
4	2.45	12 20	4 29	3.06	6 23	11.15	13 77	13 27	11.93	11.09	8 92	221 50

obtained indicate that when a sufficient concentration of ammonium sulphate is present, the nitrite production is very high and that the daily production of nitrite is limited only by the amount of ammoniacal nitrogen present in the culture, and that under uniform conditions of food, the production of nitrite is as uniform as could be expected.

The additions of ammoniacal salts are shown in figure 2.

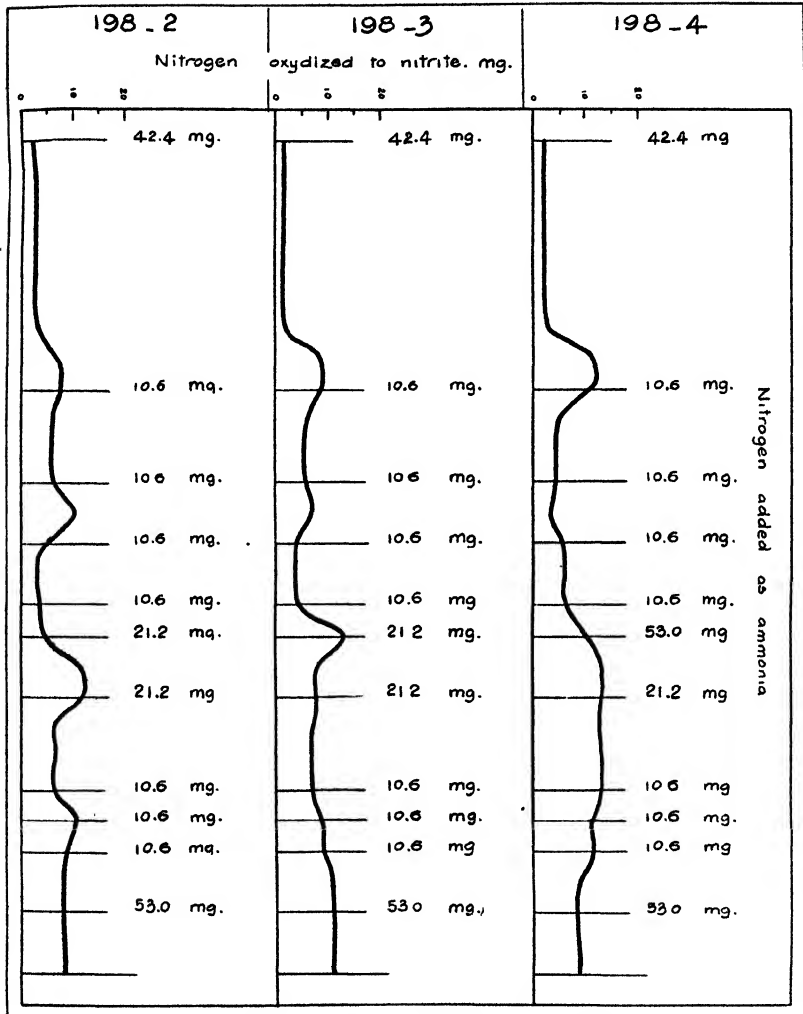


FIG. 2. PRODUCTION OF NITRATE NITROGEN (SEE TABLE 4)

Disregarding now the daily production of nitrite it is possible to obtain an index of the oxidative power of the cultures by expressing it in terms of nitrite production during the whole period of incubation. Table 5, compiled by this method, emphasizes again the strong action of the cultures undergoing a slow movement and strong aeration.

TABLE 5

Oxidative power of cultures undergoing aeration and slow movement

NUMBER OF CULTURE	LENGTH OF EXPERIMENTAL PERIOD	NITROGEN OXIDIZED TO NITRITE
	<i>days</i>	<i>mgm.</i>
186-1	14	168.92
194-1	17	173.93
194-2	17	194.49
198-2	28	187.38
198-3	28	199.05
198-4	28	221.50

MICROSCOPIC STUDY OF THE CULTURES

Although the flasks were opened very often and each sampling required some time, during which the cotton plug was removed from the neck of the flask, yet the nitrite formers held the upper hand over any foreign organism which was introduced. Photomicrograph 3 shows the state of purity of one of the cultures when observed by the microscope. Although an infecting form was occasionally observed, yet the intensive transformation of ammoniacal nitrogen to nitrite nitrogen enabled the organism of nitroso-fermentation to grow to such an extent, through the energy thus obtained, that it crowded out the infecting form, rendering its action insignificant.

The beautiful vegetation found in all the flasks studied, makes it quite plain that a strong aeration of the medium with only a moderate movement, not only furthers the oxidative power of the organism of nitrite fermentation, but also stimulates its reproductive power.

The intensive cultures above described were all derived from cultures which in an absolutely pure state grew on silicate jelly forming the colonies pictured in photomicrographs 1 and 2. A full description of the morphology of these organisms will be published in the near future.

DISCUSSION

The results reported above show that the ordinary Omelianski solution for the growth of the nitrite former, will support a very abundant growth of this organism as measured by the production of the by-product of its growth, nitrous acid (or its salts). The conditions under which this luxuriant growth was produced were (1) shallow layers of the solution, (2) slow rotary movement of the culture, and (3) a temperature of 25° to 30°C. The growths reported are far in excess of any yet reported for equal periods of time in solution cultures.

From the results of Löhnis and Green, in which case the ratio of diameter to depth in the solution layers was 90 : 1, and the depth 2 mm., it is seen that even such shallow layers are incapable of supporting a rapid nitrification. Moreover, the results secured by Barthel do not show a much stronger nitrification in aerated than in non-aerated cultures, although the continual bubblings of air through the cultures as used by Barthel may not have been wholly beneficial. It is interesting to note further that Boullanger and Massol reported good results in experiments in which, among other provisions, the cultures were changed in position with respect to a porous substance at the end of every six hours.

It would seem therefore that better aeration may not be the only factor contributing to intensive nitrification, but that change of local environment is in some way associated with it. This assumption is directly in line with the suggestions and postulations of Lucet, and throws a new light on the study of conditions favoring growth in solutions.³ Whether this change

³ The present author regrets that he was unable to obtain a recent paper by Otto Meyerhof (*Arch. ges. Physiol.* 166, 240-280 (1917)). An account of the paper to be found in *Physiol Abstr.* 2, 207 (1917) as well as in *Chem. Abstr.* 11,

of environment—if indeed it proves to be actually operative—is associated with removal of by-products or better access of mineral nutrients, or possibly some other factor, must be determined by subsequent work.

In conclusion it should be stated that work along these lines is being continued under the general direction of Dr. E. R. Allen, to whom thanks are due for kind advice and criticisms in the preparation of this paper.

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2216, (1917) states that " under favorable conditions cultures were obtained which in twenty-four hours could oxidize 4 grams $(\text{NH}_4)_2\text{SO}_4$ per litre to nitrite." Unfortunately no mention is made of what actually constitutes the "favorable conditions."

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EXPLANATION OF PLATE 1

**PHOTOMICROGRAPH 1. PORTION OF SILICA JELLY PLATE SHOWING COLONIES
WITH COLORLESS HALO DUE TO THE SOLUTION OF THE MgCO_3**

As seen against a black background. $\times 1.5$

**PHOTOMICROGRAPH 2. A COLONY ON A SILICA JELLY PLATE SHOWING THE
LOOSE STRUCTURE**

As seen against the source of light. $\times 77$

**PHOTOMICROGRAPH 3. PREPARATION FROM A CULTURE IN SOLUTION DURING
A PERIOD OF INTENSIVE NITROSO-FERMENTATION**

Stained by Winogradsky's malachite-green and gentian-violet method. $\times 1200$



1



2



3

STUDIES ON THE BACTERIAL FLORA OF THE MOUTH AND NOSE OF THE NORMAL HORSE

EDWARD E. H. BOYER

From the Laboratory of Bacteriology of the Ohio State University

Received for publication March 5, 1918

The object of this work was to determine the various species of bacteria which are normally present on the mucous membranes of the mouth and nose of the horse. The value of such a study becomes apparent when one considers that horses are subject to many diseases of unknown or doubtful etiology, such as some forms of rhinitis, laryngitis, vesicular stomatitis, etc., and that an endeavor to determine the causative factors in such diseases will invariably involve the elimination of those organisms that are normally present.

Methods. The horses used in this investigation were mares and geldings with perfectly sound and healthy mucous membranes. They included express horses, University Farm horses, and a few horses in the Veterinary Hospital.

Cultures were taken by swabbing, with sterile swabs, the mucous membranes of the hard and soft palates, tongue, cheek, gums, floor of the mouth, and nasal cavity. The swab cultures were used to inoculate tubes of melted agar, which were plated out in the regular manner. Pure cultures were obtained by "fishing" from the colonies thus grown on the plates. Each culture was subjected to the usual cultural and morphological tests, and the determination of species was aided by the use of Chester's "Manual of Determinative Bacteriology," and Matzuschita's "Bakteriologische Diagnostik."

Results. The various bacteria obtained are recorded in the following table:

Organisms present in the mouth and nose of the horse

NUMBER OF ORGANISM	NAME OF ORGANISM	MOUTH—PER CENT OF HORSES IN WHICH IT WAS FOUND	NOSE—PER CENT OF HORSES IN WHICH IT WAS FOUND
1	<i>B. buccalis</i>	4	
2	<i>B. capillaceus</i>	4	
3	<i>B. cereus</i>	4	
4	<i>B. cloacae</i>	8	
5	<i>B. coccineus</i>	8	
6	<i>B. coli</i>	12	
7	<i>B. cuticularis</i>		4
8	<i>B. detrudens</i>	4	
9	<i>B. gangraenae</i>	32	4
10	<i>B. geminus</i>	4	
11	<i>B. liodermos</i>	4	
12	<i>B. liquifaciens</i>	8	
13	<i>B. magnus</i>	12	
14	<i>B. megatherium</i>	12	12
15	<i>B. mesentericus</i>	32	12
16	<i>B. moribificans</i>		24
17	<i>B. prausnitzii</i>	28	4
18	<i>B. stellatus</i>	32	60
19	<i>B. subtilis</i>	24	16
20	<i>B. viscosus</i>	8	
21	<i>B. vulgatus</i>	12	
22	<i>Mic. aethebius</i>		4
23	<i>Mic. aurantiacus</i>	8	
24	<i>Mic. citreus</i>		28
25	<i>Mic. citreus-granulatus</i>	8	
26	<i>Mic. cremoides</i>	4	
27	<i>Mic. flavus</i>	8	
28	<i>Mic. orbiculatus</i>	24	20
29	<i>Mic. ovalis</i>		8
30	<i>Mic. pyogenes-albus</i>	12	12
31	<i>Mic. simplex</i>	4	8
32	<i>Mic. tetragenus</i>	4	
33	<i>Molds</i>	48	64
34	<i>Ps. pyocyanea</i>	8	4
35	<i>Sarcina aurantiaca</i>	12	12
36	<i>Sarcina flava</i>		8
37	<i>Sarcina lutea</i>	24	24
38	<i>Staph. epidermidis albus</i>	16	16
39	<i>Streptothrix chromogena</i>	32	88
40	<i>Strep. pyogenes</i>	8	4
41	<i>Strep. vermiformis</i>	8	4
42	Not determined (a).....	8	

NUMBER OF ORGANISM	NAME OF ORGANISM	MOUTH—PER CENT OF HORSES IN WHICH IT WAS FOUND	NOSE—PER CENT OF HORSES IN WHICH IT WAS FOUND
43	Not determined (b).....	8	
44	Not determined (c).....	4	
45	Not determined (d).....	4	
46	Not determined (e).....	4	
47	Not determined (f).....	4	
48	Not determined (g).....	12	
49	Not determined (h).....	8	
50	Not determined (1).....		8
51	Not determined (2).....		16
52	Not determined (3).....		16
53	Not determined (4).....		8

It will be observed that twelve organisms are "not determined." i.e., their cultural characteristics did not correspond to those of any species given by Chester or Matzuschita. Most of these bacteria were large, rod-shaped, spore bearers, all but one being Gram positive. They differed but slightly from some of our common saprophytes. To give such forms a new name, and to place them as entirely new species would be entirely unwarranted. They might better be considered, at present, as new strains; the result perhaps, of a slightly different environment.

CONCLUSIONS

1. The nose and mouth of the normal horse harbor a large flora of micro-organisms.
2. The great majority of these organisms are harmless saprophytes.
3. Occasionally present are a few bacteria which may become pathogenic under certain conditions.

BACTERIOLOGICAL NOTES

I. GAS PRODUCTION BY BACT. PULLORUM

II. BACT. PULLORUM INFECTIONS IN ADULT BIRDS

III. RELATION BETWEEN SUCROSE-FERMENTATION AND IMMUNIZING POWER BY B. AVISEPTICUS

PHILIP HADLEY, DOROTHY W. CALDWELL AND BERTHA M.
HEATH

*From the Agricultural Experiment Station of the Rhode Island State College,
Contribution 243*

Received for publication March 10, 1918

I. GAS PRODUCTION BY BACT. PULLORUM

Bact. pullorum (Rettger) is typically a gas-producing organism. Occasionally, however, an anaerogenic strain is observed. Observations made by the writers may throw light on this phenomenon.

In certain tests made in connection with another investigation, certain strains of this organism which had been isolated from eggs or from chicks dying of bacillary white diarrhea or from adult stock, and which had been maintained for some years in the laboratory, were grown in extract and in infusion glucose broths in Smith fermentation tubes. At the same time several strains of *B. gallinarum* (E. Klein), the causative agent of so-called fowl typhoid, were grown in the same manner. The results were as shown in Table 1.

From these data it appears that many strains of *Bact. pullorum* that do not produce gas in extract media may produce gas in infusion media; also that when any amount of gas is produced in extract media a larger amount is produced by the same strain in infusion media. Gas-production in sugar media is apparently strongly influenced by environmental factors independent of the presence of the specific fermentable sugar.

TABLE 1

Gas-production by strains of Bact. pullorum and of B. gallinarum in glucose extract broth and in glucose infusion broth

STRAIN NO.	EXTRACT BROTH				INFUSION BROTH			
	24-hour	48-hour	72-hour	96-hour	24-hour	48-hour	72-hour	96-hour
12	0	0	0	0	0	B	B	B
13	B	B	10	10	5	15	20	20
14	0	0	0	0	8	16	20	20
15	B	B	5	5	3	12	15	15
17	0	0	0	0	0	B	3	3
18	0	0	0	0	B	8	10	10
34	0	0	0	0	0	B	3	3
56	0	0	0	0	3	13	15	18
59	0	0	0	0	0	0	0	0
93	0	0	5	5	B	8	12	12
99	0	0	5	5	0	B	8	8
88*	0	0	0	0	0	0	0	0
115*	0	0	0	0	0	0	0	0
116*	0	0	0	0	0	0	0	0
102†	0	0	0	0	0	0	0	0
118†	0	0	0	0	0	0	0	0
162†	0	0	0	0	0	0	0	0

* *B. gallinarum*.

† *Bact. pullorum* from infections in adult stock.

II. BACTERIUM PULLORUM INFECTIONS IN ADULT STOCK

Although cultures of *Bact. pullorum* coming from infected chicks or infected eggs are aerogenic, the writer has been able to demonstrate that anaerogenic strains of this organism do exist. It is a noteworthy circumstance, however, that these strains came in every instance from infections in *adult stock*. The writers have already in another place (1918) presented in detail the circumstances attending an epidemic among adult stock in which *Bact. pullorum* was the causative agent. At the same time reference was made to two other epidemics in adult fowls caused by this organism.

Jones (1913) some years ago discussed an epidemic in adult stock, coincident with a serious infection of chicks on the same farm which was said to be due to *Bact. pullorum*. Since, however at the time of Jones's work, methods were not at hand for the

differentiation of *Bact. pullorum* and *B. gallinarum*, it is not certain which organism was concerned in this case described by Jones. The present writers therefore wish to make it a matter of record that in three widely separated epidemics among adult fowls, each involving a considerable mortality, they have isolated in pure culture, and as the only micro-organism present in cultures from the blood and organs, three bacterial strains conforming to *Bact. pullorum* in all essential respects except that they are not aerogenic. It should be added that the clinical features of the disease, as well as the pathological findings at autopsy, differed in no important respect from those of fowl typhoid. It should also be added that in the ovaries and in the eggs from birds dying in one of the epidemics mentioned, aerogenic strains were also found; but these aerogenic organisms were not found in the other organs of the body, in the heart blood or in the pericardial or pleural exudates. These findings have led the writers, as elsewhere stated, to postulate two types of *Bacterium pullorum*: (1) *Bact. pullorum* α , aerogenic and found only in infections of young stock, or as a latent ovarian infection in adult stock; (2) *Bact. pullorum* β , anaerogenic, and observed only as an agent of active infection in adult stock. Slight fermentative differences between these sub-types may also exist.

An explanation of this diversity of type, together with the difference of selective action in the tissues of the fowl is not at present possible. The questions naturally arise: (1) Are both types present in the intestinal tract of fowls, and does the beta type produce generalized infection because of a difference in aggressivity? Or, (2) may the alpha type give rise to the beta type during the progress of ovarian or intestinal infection, as a result of adaptations, or as a result of a selection of anaerogenic mutants? Is *Bact. pullorum* an instance of *B. gallinarum* in the making?

III. CORRELATION BETWEEN SUCROSE FERMENTATION AND IMMUNIZING POWER OF *BACILLUS AVISEPTICUS*

One fundamental fermentative characteristic of *B. avisepticus*, and one by which it can be most readily differentiated from members of the fowl typhoid group, is acid-production in sucrose media. The fermentative tests performed by the writers, including a series of fowl cholera strains, showed all, except one, to be sucrose positive. This was strain 52, a culture referred to frequently in earlier publications (1912) as unique in the following points: (1) it is avirulent for adult rabbits, (2) it possesses marked immunizing power against infection with virulent

TABLE 2

Difference of reaction in infusion broth, after two days, of different strains of B. avisepticus. (The percentages give the actual amount of acid produced after the acidity of the control tube has been deducted)

STRAIN NO.	DATE OF TEST		
	January 30, 1918	February 8, 1918	February 18, 1918
48	2.6	2.2	3.5
52	0.2	0.2	0.7
62	2.6	2.5	3.4
83		2.3	3.1
84b		3.3	3.2
91		2.3	2.5
Control	0.5	0.5	0.0

strains.¹ The following tabulation gives the results of titrations of two-day infusion sucrose broth cultures made at different times and in different lots of media (Table 2).

From these data it appears that strain 52 is persistently negative in sucrose broth while the other strains give high acidity. The writers find reported in the literature no other instance of a sucrose-negative strain of *B. avisepticus*. Thus culture 52 is the only sucrose-negative strain described and also

¹ These results of the present writer have been confirmed in several other laboratories, the most complete tests having been made by Gallagher (*Jour. Amer. Vet. Med. Assn.*, 1916-17, 50, 708-728).

the only immunizing strain. Whether this relation of biochemical features is coincidental or whether it is of some definite significance in immunity-production remains a question.

SUMMARY

1. Gas production by *Bact. pullorum* may depend upon whether the cultures are grown in glucose extract or in glucose infusion broth. Propagating cultures for many years on artificial media does not cause them to lose their gas-producing ability.

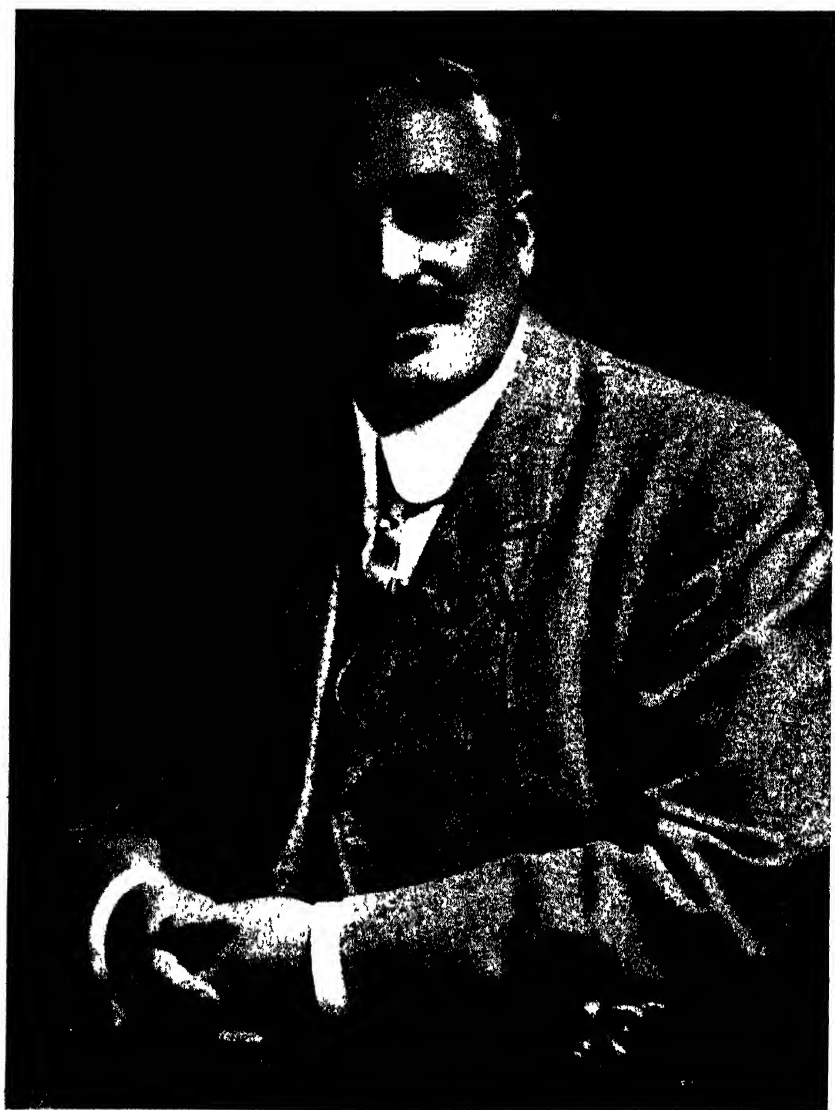
2. Although *Bact. pullorum* isolated from epidemics of bacillary white diarrhea in young chicks, or from infected eggs, is aerogenic, there exist also anaerogenic strains which, in all the cases in which they have been observed, have been isolated from adult fowls experiencing acute or sub-acute infections simulating fowl typhoid in both clinical symptoms and pathological alterations in the tissues.

3. Since the anaerogenic quality is thus positively correlated with source, the writer proposes tentatively to postulate for *Bact. pullorum* two sub-species: (1) *Bact. pullorum* α , aerogenic, pathogenic for chicks; and (2) *Bact. pullorum* β , anaerogenic and pathogenic for adult stock only.

4. *B. avisepticus* typically produces an acid-fermentation in sucrose. Among several strains examined, however, one (strain 52) was negative for this sugar. In this instance the absence of sucrose fermentation was correlated with the ability to produce in rabbits resistance against powerful infections with virulent cultures, an ability possessed by no other strain. The question is raised as to the significance of the fermentative reaction in relation to immunity production.

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Frank Fairchild Westbrook

FRANK FAIRCHILD WESBROOK

H. W. HILL

Frank Fairchild Wesbrook was born July 12, 1868, at Oakland, Ontario, Canada, and died, at the age of fifty, on October 20, 1918, at Vancouver, B. C., after an illness of a year's duration.

From the year 1913 and until the time of his death he was President of the University of British Columbia.

He leaves a widow, Mrs. Annie Wesbrook, and a daughter, Helen Wesbrook, the latter in the senior year of the Arts College.

President Wesbrook took his Bachelor's and Master's degrees at the University of Manitoba in 1887 and 1888, and the degrees of M.D. and C.M. in 1890 from that University and McGill College; while in later years the Universities of Manitoba, Toronto and Alberta conferred upon him the degree of LL.D. During 1891-1893, he served as Professor of Pathology at the University of Manitoba; thence he went to Cambridge University in 1893-1895 as John Lucas Walker student, where he engaged in research work in pathology and served as a University tutor under Gaskell.

In 1895 he was called to the directorship of the Department of Pathology, Bacteriology and Hygiene at the University of Minnesota, filling this position as professor throughout his connection of eighteen years with this University.

Dr. Wesbrook's chief work was in Bacteriology—particularly as related to public health. With O. McDaniel and L. B. Wilson he published the famous "Types of Diphtheria Bacilli" which did so much to simplify, and make accurate, diphtheria diagnosis. He was one of the earliest and most foresighted investigators and advocates of chlorine sterilization of water supplies, which has become an immensely important factor in public health.

He was Director of the Minnesota State Board of Health Laboratories until 1913 and for two periods was a member of the Minnesota State Board of Health.

In 1906 he was appointed to the Deanship of the Medical School of the University of Minnesota and efficiently administered his trust until he was invited to the Presidency of the University of British Columbia and resigned his position at Minnesota.

He threw himself ardently into the development of the new University at Vancouver and throughout the war was especially concerned in the training of young men and women for war service and for the coming duties of reconstruction.

Dr. Wesbrook was an active worker in the interests of public health and for many years has been recognized in the public health councils of this country and of Canada. He was Chairman of the Laboratory Section of the American Public Health Association in 1901, and was elected President of that Association in 1905, serving during the Boston meeting. He was a charter member of the American Society of Bacteriologists. In 1912 he was chosen President of the section on State and Municipal Hygiene of the International Congress of Hygiene and Demography and for ten years served as a member of the Advisory Board of the Hygienic Laboratory at Washington, D. C.

His powerful physique, splendid bearing and great cordiality made him an impressive and attractive figure everywhere.

His demise excited general regret, and no one who knew him but feels a great personal, as well as professional, loss; the world was richer for his presence and poorer for his departure.

AGGLUTINATION¹

R. E. BUCHANAN

From the Bacteriological Laboratories, Iowa State College, Ames, Iowa

The address of a retiring president of a scientific society at this time might well be devoted to a discussion of the relationship of the science in question to the conduct and winning of the war. Certainly bacteriology has played a major rôle in this drama. Its contributions have been of fundamental importance. Therefore, notwithstanding the fact that there are many who have been in far closer contact with the war triumphs of our science than have I, it is probable I should have essayed the task, had it not been for the events of the past few weeks. The war, we confidently believe, has been won. May we not, then, address ourselves to the pursuits of peace instead?

I shall confine my remarks to an entirely different phase of our science, namely, the phenomenon of agglutination, particularly to certain physico-chemical conceptions and their bearing upon a theory of agglutination.

For many years the terminology and conceptions of Ehrlich as they related to the phenomenon of agglutination have held sway in our texts and have in general proved fairly adequate for an introductory or rather casual explanation of this phase of immunology. They have served as guides in our discussions of the differentiation and recognition of bacteria by specific sera, and of the diagnosis of disease by use of specific bacteria. In some quarters, perhaps as a natural result of our anti-Teutonic bent in the Great War, there are signs of revolt against the dominance of such expressions. Witness a recent statement by Moore (1917) in the *British Medical Journal*:

¹ Presidential Address delivered before the Society of American Bacteriologists at its Twentieth Annual meeting, December 27, 1918.

Progress in our knowledge of the biochemistry of immunity will be delayed so long as the modes of thought connoted by the series of *ad hoc* terms first introduced by Ehrlich and since reduplicated by others, are allowed to dominate the clear view that the laws of immunity are the laws of reaction by adsorption in colloidal solution. There is nothing new or ever was in the side chain theory except the fantastic terms. . . . The *pioneer* work on immunity which has carried us so far forward in many a domain of pathology and bacteriology was that of Bordet, Gengou and others of the French School; it was burdened from Germany with the intolerable weight of a useless philosophy of jangling terms for a type of reaction well known in colloidal chemistry. . . .

Further,

All the laws of immune reactions can be illustrated by colloidal systems which are not and never were living and the preservation of a special set of terms is redundant.

Also in the same vein the statement by Roberts (1917),

I wish to ask whether it is not time to simplify the theory of immunity. Much modern work seems intended to support "orthodox Ehrlichism," though by the multiplication of sub-hypotheses its confusion grows worse confounded. The terms employed in general physiology should thus be sufficient for bacteriology, and observers of fresh phenomena ought to be chary of coining new words. Their hasty multiplication usually implies some additional hypothesis. It is characteristic of a false explanation to require an increasing number of sub-hypotheses while a real one abolishes a multitude of superfluous terms and, displaying a phenomenon as the "functions of known variables, by such a disclosure is essentially a simplification."

These caustic criticisms to the contrary notwithstanding, it probably will be long before the Ehrlichean conception of receptors of the second order passes from our texts on bacteriology and immunity. However, the phenomenon of agglutination has been found to be complex, so complex that the older explanations and conceptions fit the facts only relatively and poorly. We hear now of co-agglutination and conglutination, the fixation of complement in agglutination, of acid or hydrogen ion agglutina-

tion and precipitation. When we expand and revamp the Ehrlichean terminology to fit the new facts we are in danger of being lost, in a forest of verbiage. Physical, colloidal, and bio-chemistry have come to our rescue, and have contributed facts which will assist us in formulating a better and more consistent conception of the agglutination phenomenon. I have derived much pleasure from a review of some of this work and an attempt to correlate and systematize it into a well coördinated theory, drawing in part upon experimental results of my own for illustration. Other than this illustrative material there is little that is new in my discussion.

A. HISTORICAL

Investigators who followed the initial discovery of the phenomenon of bacterial agglutination by Gruber and by Widal may be divided into three groups; one, with Ehrlich as leader, studied the phenomenon of serum agglutination quite intensively, and built up around their observations a relatively complex portion of the side chain theory of immunity. Ehrlich, Eisenberg, Volk, Wasserman and others introduced a formidable terminology for the phenomena observed. Much of this terminology proved valuable from both theoretical and practical points of view, but there was relatively little effort to base the theories upon well recognized laws of colloid chemistry.

A second group of investigators interested themselves in efforts to work out methods of bacterial identification by agglutination produced by substances other than the homologous antisera. The first of these workers, apparently, was Malvoz (1897, 1899) who studied the flocculating effect of many chemicals, and concluded that the typhoid bacillus was specifically agglutinated by formaldehyde. Beco (1899) and others, however, showed this reaction to be non-specific. This type of investigation was also pursued by Bossart (1898) and by Sabrazes and Banzierés (1899), who found many substances which would cause bacterial agglutination, but none which made practicable the satisfactory differentiation of species. Michaelis (1911) concluded that

the typhoid bacillus in suitable suspension shows a specific agglutination in a definite hydrogen ion concentration in a suitably buffered solution. Beniasch, (1911) extended this observation to a large number of other bacteria, concluding that acid agglutination could be used as an aid in the recognition of many species. Favorable results were secured by many other investigators as Rost (1911), Jaffe (1912), Poppe (1912), Schidorsky and Rein (1912), Stepanoff (1912), Grote (1913), Sgalitzer (1913), and Gillespie (1914). Crendiropoulo and Amos (1904) showed that certain salts favored agglutination. Liefmann (1913) believed it possible to differentiate bacteria by a process of salting out similar to that used with proteins.

The third group of investigators have approached the subject from the standpoint of physical or colloidal chemistry, and have attempted to correlate results secured in bacterial agglutination with similar non-specific phenomena in chemistry. These writers do not use the Ehrlichean terminology, and their results and conclusions do not generally find place in our texts. It seems demonstrable however, that they have pursued the course which is leading to a logical and satisfactory explanation of the immunity phenomena. Among them Bordet (1896) clearly recognized and proved that in specific agglutination there are two distinct phases, first, the impression or sensitization of the bacterial cell by the agglutinins and second the precipitation or agglutination of the cells due to the action of salts. He emphasized the fact that this agglutination was homologous with the flocculation of colloidal solutions by salts. His results were extended and confirmed by Joos (1902). Two years later three important contributions were made, emphasizing the homology of the agglutination reactions with colloidal phenomena. Bechhold (1904) carried out a well planned series of tests with mastic and with bacteria both sensitized and unsensitized. His work is so conclusive and gives such excellent illustrative material for class and laboratory demonstration that it is surprising that so little use has been made of it. He investigated the activity of various ions, showed that bacterial cells carry electric charges, determined the agglutinating limits of many substances, and demonstrated both

regular and irregular series. Landsteiner and v. Jagic (1904) called attention to the analogy between the action of colloidal silicic acid and the immunologic reactions. Neisser and Friedmann (1904) duplicated much of the work of Bechhold and reached similar conclusions. Zangger (1905) also emphasized the complete agreement between the action of immune bodies and colloids. Landsteiner and Stankovic (1906) studied the adsorption of proteins and the binding of agglutinins. In the same year Porges (1906) carried on numerous experiments. In more recent years contributions have been made through the work of Millen (1909), Bordet and Streng (1909), Dreyer and Douglas (1910), Michaelis (1911), Beniasch (1911), Krumwiede and Pratt (1912), Michaelis and Davidsohn (1912), Ostwald (1912), Dean (1912), Chick and Martin (1912), Landsteiner (1913), Ostwald (1913), Schmidt (1913), Tullochs (1914), Walpole (1914), Hofmann (1914) and Priestley (1917). These workers have placed our knowledge of bacterial agglutination on a relatively sound basis.

B. WHY DO BACTERIA AGGLUTINATE?

Before undertaking a consideration of the question, why do bacteria agglutinate, it may be well to ask, why do bacteria *not* agglutinate? An observation of a hanging drop of bacteria will usually show either active motility or active Brownian movement. Cells constantly collide, or at least approach each other closely, then they move apart, apparently repulsing each other, and remain quite uniformly distributed over the field. The factors which may have to do with agglutination are the repulsion of the cells for each other, the vigor of their movements whereby they collide or enter each others sphere of effective influence, and the attraction which the cells may possess for each other. Agglutination occurs whenever the forces tending to draw these particles together are more powerful than those of repulsion.

Let us first consider the factors which determine the repulsion of bacterial cells for each other. It has long been known that particles suspended in water usually bear an electric charge.

When placed in an electric stream they generally move toward the anode, that is, they bear a negative charge; in a few cases the charge is reversed and they move to the cathode. Hardy (1899) showed that the particles of a colloidal suspension of boiled egg-white moved to the anode, but that the direction could be reversed by the presence of suitable electrolytes. This observation was extended to bacterial suspensions by Bechhold (1904) who found that bacteria whether heated or unheated wandered to the anode. This fact has been abundantly confirmed by other investigators since. Among these were Neisser and Friedmann (1904) Porges (1906), Porges and Prantschoff (1906), Michaelis (1909), Beniasch (1911), Schmidt (1913), Landsteiner (1913), Arkwright (1914), and Priestley (1917). Apparently suspensions of bacteria as ordinarily grown are negatively charged and the mutual repulsion of the cells may be due to these charges.

The occurrence of the phenomenon of flocculation or agglutination is in itself evidence that there must exist a force of attraction, under certain circumstances at least. This force is probably surface tension. If the bacterial cells do not repel each other, nor attract each other *per se*, the phenomenon of surface tension explains the fact that they remain together.

It is evident then, that we have at least a partially adequate explanation of why bacteria under certain conditions remain in suspension, and why under other conditions they may cling together. We may regard the similar electric charge as constituting the repulsing agency, and surface tension as the attracting agency. A study of the agglutination phenomenon then resolves itself into a consideration of the means whereby these two forces may be modified, increased or diminished. Agglutination occurs whenever the similar electric charges are decreased to amounts such that they will no longer overcome the pull of surface tension. Or conversely, surface tension may be increased until it overcomes the dispersion effect of the similar charges. What are the factors which govern the intensity of these forces?

C. CATAPHORESIS

First let us consider methods which may be used to modify or even reverse the charge upon bacterial cells. To Hardy (1900) we owe a knowledge of the fact that particles of denatured proteins may be deprived of their charge, that is, rendered isoelectric with the solution, or may have their charges reversed by the addition of electrolytes. Particularly marked was found to be the activity of acids, alkalies and salts. After suitable adjustments it was found that the particles moved neither to anode nor cathode, and the particles flocculated. The addition of further small quantities of either alkalies or acids caused the particles to disperse and acquire the property of traveling in the electric field. It is evident then that with certain suspensoids electrolytes will cause flocculation when the particles are made isoelectric. Is the same true of bacterial suspensions? Beniasch (1911) has shown that a change in hydrogen ion concentration to a point where typhoid bacteria agglutinate does not necessarily neutralize the charge on the bacterial cells, for when showing acid agglutination they may still pass to the anode. The apparent discrepancy possibly arises from the fact that bacteria do not consist of a single colloid and this agglutination may represent the isoelectric point of some one of the constituents. Arkwright (1914) indeed found that in *Bacillus typhosus* there are two distinct substances agglutinable by acids, and at least one of these flocculates at its isoelectric point. We may safely conclude that the charge carried by a bacterial cell, or at least by its various components, can be altered by additions of various substances to the solution.

It may be accepted then that the presence of electrolytes may modify the charge on a bacterial cell. But how? Apparently by the adsorption of ions or particles bearing an opposite charge. Conversely, the charge on the cell may be due to the various substances adsorbed, frequently to the ions. It is necessary that there be such an affinity between the positive cation, for example, and the negative bacterial cell that the latter will adsorb the former. The dispersive force is thus gradually neutralized.

D. DENATURATION OR SENSITIZATION

It should also be noted that the ability of a particle to adsorb an ion may be modified. Proteins in particular may be changed from a form difficult to flocculate in any electrolyte concentration to one flocculated by even slight concentration. Hardy (1899) pointed out that coagulation or precipitation of proteins as a result of hot water involved two quite distinct processes; first, a fundamental change in the protein which modified its characteristics markedly, and second the agglutination of these modified particles under the influence of electrolytes. This transformation of a protein he termed the process of denaturation. Apparently, then, it is possible to modify the adsorptive powers of particles for ions. When this adsorptive power is increased we may speak of the process as one of denaturation, or when dealing with bacteria, as sensitization.

Tentatively we may classify the methods of denaturation or sensitization under two heads; first, a change in the chemical constitution or atomic arrangement, such as that produced by heat in presence of water or the action of some chemical; second, a change due to adsorption of some substance either a colloid or an electrolytic ion which in turn modifies its adsorptive powers. Agglutination may be produced by sensitization or denaturation of bacteria so that they will adsorb substances which will so modify their electric charges that the dispersive forces are weaker than the attracting forces.

E. FACTORS INFLUENCING AGGLUTINATION

1. Nature and composition of antigen

The nature and composition of the antigen are important factors influencing agglutination. A suspension of bacteria as ordinarily prepared for agglutination tests is a very complex mixture. The bacterial cells themselves constitute a suspensoid, and each bacterial cell is in itself a colloid mixture. It is apparent that these various constituent colloids may behave in a sense independently, and that therefore, the bacteria may

show distinct zones of agglutination. Table I gives an example of this for meningococci. It will be noted that the particular antigen shows flocculation at PH values of 3.0 and 5.0. This phenomenon was apparently first shown for *B. typhosus*, by Arkwright (1914) who found one zone of specific agglutination,

TABLE I

Effect of variation in concentration of antigen on agglutination by hydrogen ions. Suspension of washed meningococci in distilled water. Turbidity of suspensions given in silica standard equivalents. 56° for thirty minutes. Twenty hours at room temperature. 4 = Complete agglutination. 0 = No. agglutination

PH VALUES	DILUTION OF ANTIGEN		
	1000 p. p. m.	500 p. p. m.	250 p. p. m.
2.2	3	3	2
2.4	4	4	4
2.6	4	4	4
2.8	4	4	4
3.0	4	4	4
3.2	4	4	4
3.4	4	4	4
3.6	3	4	3
3.8	3	2	2
4.0	2	2	0
4.2	1	0	0
4.4	0	0	0
4.6	0	0	0
4.8	2	1	0
5.0	3	2	1
5.2	1	1	0
5.4	0	0	0
5.6	0	0	0
5.8	0	0	0
6.0	0	0	0

at an hydrogen ion concentration of $3.6 \times 10^{-5}N$ and another, apparently non-specific, at a concentration of $1.1 \times 10^{-3}N$.

It is probable that in spite of the complexity of the bacterial cell a comparatively small number of colloids are responsible for the reactions incident to agglutination.

Marked differences are to be observed in the agglutination of capsulated and non-capsulated bacteria due apparently to the

inhibiting influence of the capsular material itself. This is particularly well shown in the difficulty encountered in producing agglutination of Friedlander's bacillus. That this difficulty is due to the capsule was shown by Porges (1905), who succeeded in agglutinating the organism after hydrolysis of the capsule. Streit (1906) found that Friedlander's bacillus grown at 8°C. was drier, produced less capsular material and was more easily agglutinable than when grown at blood heat. Beham (1912) showed that agglutinin formation takes place in animals injected either with capsulated or non-capsulated strains, but can best be demonstrated with the occasional capsuleless cultures developed.

Inagglutinable strains of non-capsulated bacteria which are usually readily agglutinable have been frequently described. Weeney (1899) reported decided differences in agglutinability among typhoid races, particularly those from bile. Rodet (1899) isolated from the spleens of three typhoid cadavers bacteria which were not agglutinable when first cultivated, but finally became so. This experience was duplicated by Sacquépée (1901). Bail (1902) claimed that typhoid cells from the peritoneal exudate of a guinea pig showed decreased agglutinability. Cole (1904) found that inagglutinable bacteria bind less agglutinin than agglutinable bacteria. Kirstein (1904) cites many instances of inagglutinable bacteria. Ficai (1912) emphasized that freshly isolated vibrios do not agglutinate as well as the older laboratory strains. Reimer (1913) demonstrated a definite diminution of agglutinability in typhoid grown on alkaline agar. McIntosh and McQueen (1914) found that an inagglutinable strain of typhoid had normal antigenic powers in production of anti-typhoid serum, and that it absorbed agglutinins though it did not agglutinate.

On the other hand bacterial cultures are frequently noted that are hyper-agglutinable. Kirstein (1904) found that typhoid bacilli when grown on asparagin agar became spontaneously agglutinable in salt solution. Neisser and Friedmann (1904) and Bechhold (1904) concluded that spontaneously agglutinating bacteria lack some substance, perhaps a protein, that is responsible for the inhibition of flocculation in normal cultures.

Friedberger and Lürssen (1905) found spontaneous agglutination to be identical in appearance with serum agglutination. They also noted the important fact that it takes place only in the presence of salts, not in distilled water. Porges and Prantschaff (1906) showed that bacteria that were spontaneously agglutinable still combined with agglutinin. Porges (1906) also showed that an excess of agglutinating serum inhibits the agglutination of spontaneously agglutinable strains. McGregor (1910) found that meningococci grown for more than forty-eight hours became easily agglutinable, flocculation occurring in physiological salt solution or in normal serum. Kabeshima (1913) noted that old cholera bacilli frequently showed spontaneous agglutination. The agglutinins were adsorbed by these cells as well as by normal ones. Dilution of the salt prevented non-specific or spontaneous agglutination. Verzai (1917) found a typhoid organism in urine which agglutinated spontaneously in physiological salt solution. Even after six weeks cultivation it still showed this peculiarity. He showed, however, that specific serum agglutination could be secured with a salt solution one-fourth the strength of physiological salt solution.

The antigen will contain varying quantities of products of growth and metabolism, and frequently also the products of more or less complete cell autolysis. These are the substances of import in the specific precipitation test as differentiated from agglutination. It seems probable that in some cases at least they are identical with the agglutinable constituents of the cells themselves. This is borne out by the experiments of Arkwright (1914). It is possible to wash bacteria until they lose a large part of their specific agglutinability.

The conditions under which bacteria are grown, the exact method of preparing the antigen, together with unexplained variations in the bacteria themselves, all may have their effect upon agglutinability. In a few cases this may even influence the development of agglutinins in the immunized animal. The experiments by Bordet and Sleeswijk (1910) on pertussis may be cited. Gay and Claypole (1913) found that so-called blood and bile strains of typhoid did not agglutinate with anti-typhoid

serum, although they absorbed it. They agglutinated however with the homologous antiserum.

Concentration of the antigen is of marked importance in the agglutination phenomenon. As will be noted later, this effect is manifested either when the precipitating agency is an electrolyte or a colloid, though in different ways. Porges (1906) with a suspension of typhoid secured partial agglutination in undiluted antigen by 30 per cent saturation with $(\text{NH}_4)_2\text{SO}_4$, but not in higher dilution. Examples of this effect with a men-

TABLE 2

Agglutination of meningococcus by calcium chlorid. Effect of dilution of antigen. Suspension of washed meningococci in distilled water. Incubated at 56°C. for twenty hours

DILUTION OF CaCl_2	STRAIN OF ANTIGEN					
	55		59		60	
	1000 p. p. m.	500 p. p. m.	1000 p. p. m.	500 p. p. m.	1000 p. p. m.	500 p. p. m.
M/2	0	0	0	0	0	0
M/4	0	0	0	0	0	0
M/8	0	0	0	0	0	0
M/16	0	0	0	0	1	0
M/32	2	0	0	0	3	3
M/64	3	2	0	0	4	4
M/128	3	2	0	0	4	4
M/256	1	0	0	0	4	2
M/512	0	0	0	0	1	0
M/1024	0	0	0	0	0	0
M/2028	0	0	0	0	0	0

ingococcus antigen are illustrated in table 1 with varying hydrogen ion concentration and in table 2 with CaCl_2 . With cerous nitrate, in dilution of 1000 parts per million complete agglutination occurred in two ranges, from M/20 to M/40 and from M/640 to M/2560 and in 250 parts per million complete agglutination occurred at M/20 and in the range M/2560 to M/5120 (see table 3). These facts were well illustrated by Buxton and Rahe (1909). These investigators differentiated clearly between two distinct types of flocculation of colloids; the electrolyte type in which dilution of the colloid has no effect upon the flocculation

limits, and there is no relation between the concentration of the electrolyte and the concentration of the colloid; and the colloidal type resulting from the flocculation of one colloid by another of opposite sign, in which the flocculation limits are dependent upon the relative concentration of the two colloids, i.e., if either colloid is much in excess there is no flocculation. Miller (1909) observed that the zone of inhibition of sera containing so-called agglutinoïd varies with density of the bacterial suspension, the more concentrated the antigen the lower the dilution of the

TABLE 3

Agglutination of meningococcus by cerous nitrate. Effect of dilution of antigen. Suspension of washed meningococci in distilled water. Incubated at 56°C. for twenty hours

DILUTION OF Ce(NO ₃) ₃	STRAIN OF ANTIGEN					
	56			60		
	1000 p. p. m.	500 p. p. m.	250 p. p. m.	1000 p. p. m.	500 p. p. m.	250 p. p. m.
M/20	4	4	4	4	4	4
M/40	3	3	1	4	4	3
M/80	2	2	1	3	2	1
M/160	0	0	0	0	0	0
M/320	0	0	0	0	0	0
M/640	4	0	0	0	0	0
M/1280	4	4	0	4	4	0
M/2560	4	4	4	4	4	4
M/5120	0	4	4	0	4	4
M/10240	0	0	0	0	0	3
M/20480	0	0	0	0	0	0

serum in which agglutination will occur. He concluded that this similarity to colloid flocculation rendered unnecessary the postulation of the existence of agglutinoids.

Beniasch (1911) calls attention to the fact that Hofmeister (1888) found a definite relationship between the size of the precipitating doses of the light metals and the dilution threshold characteristic of the protein itself. The minimal precipitating doses of such salts decreased with diminution of the concentration of the protein, while the maximal dose remained the same for all concentrations. Porges has shown a similar relationship for bacterial suspensions.

2. Adsorption of other colloids

It may be emphasized at this point that there are two somewhat variant current conceptions of bacterial agglutination. According to one view, bacterial cells determine the agglutination, they themselves repel or attract. The other conception is that a precipitation of some extraneous substance occurs, and the bacteria are carried down mechanically. It is not improbable that both conceptions are necessary to a complete explanation of the phenomenon under differing conditions. From the foregoing discussion it is apparent that the phenomenon of adsorption is in either case quite fundamental in any consideration of agglutination.

a. Colloids which agglutinate per se. Attention has been called to the fact that antigen may adsorb either colloids or electrolytic ions or both. Let us first consider the adsorption of colloids.

The adsorption of colloids by antigen may influence the course of agglutination in any one of several ways. It may favor the agglutination directly or indirectly. It may cause agglutination *per se*, it may increase the adsorption of some other colloid, or it may increase flocculability by salts and other electrolytes. In some cases it may have the opposite effect, decreasing the adsorption of other sensitizing colloids or flocculating electrolytes.

Numerous examples of colloids which agglutinate bacteria *per se* have been described in the literature. One well marked characteristic of the agglutination by the interadsorption of two colloids may be noted. An excess of either colloid beyond a definite proportion interferes with or prevents agglutination. If one colloid, as antigen, be diluted, the precipitating colloid must also be diluted. This gives rise to a so-called irregular series of Bechhold. Among the substances which have been found to flocculate bacteria in this manner are certain of the aniline dyes, hydroxids of some metals, and possibly certain sera. Aniline dyes in aqueous solution in general behave as colloids and have been used for bacterial agglutination by sev-

eral writers. Blachstein (1896) suggested the use of chrysoidin as a specific precipitant for the cholera vibrio, though Engel (1897) found its action not to be specific. Malvoz (1896) found that *B. typhosus* was not agglutinated by this dye, though he notes that safranin and vesuvin do produce agglutination in dilutions as high as those shown by a potent serum. McIntosh and McQueen (1914) found that serum-agglutinable and non-agglutinable strains of typhoid were agglutinated by safranin in dilutions up to 1 in 3000.

The hydrosols of metallic hydroxids usually agglutinate bacteria, as well as other hydrosols bearing opposite charges. Biltz (1904) arranged a large number of hydrosols in two columns. When mixed in proper proportion those of one column would agglutinate those of the second. He also showed that the same type of irregular series occurs with bacteria and serum. It had previously been noted that dilute solutions of salts of iron, aluminium, cerium, etc., showed the characteristic colloidal irregular series as a result of the hydrolysis of the salt.

There are occasional records of other colloids showing an agglutinating action. For example, Trumpp (1898) succeeded in agglutinating cholera vibrios with various gum and starch solutions. It has already been noted that Bordet (1899) first showed the action of the agglutinin to be a sensitization of the cell to the action of salt. There is some evidence however, that the agglutinin may itself cause agglutination. For example, Porges (1906) dialyzed both typhoid bacteria and a potent anti-serum until they were chlorin free and found agglutination would occur with a 1-10 dilution of serum but not in higher dilution unless salt was present. A similar fact is shown in chart I, though it is probable in this case that the undialyzed immune serum contained sufficient salt to cause agglutination of the sensitized cells.

Apparently the so-called specific agglutinins are colloids, which are taken up by bacteria in accordance with the laws of adsorption. Dreyer and Douglas (1910) studied the absorption of agglutinins by bacteria and the application of physico-chemical laws thereto. They determined that if an agglutinating

serum in different concentrations is treated with a constant number of bacteria, the amount of bound agglutinin from successive increments of serum progressively decreases and finally sinks to zero.

The amount of adsorption of one colloid by another may be markedly influenced by the presence of other substances in solution. In some cases it is possible to bring adsorbed substances back into solution by the addition of proper solutes, and thus to demonstrate the reversibility of the union. This reaction has been well worked out for hemolytic amboceptor by Kosakai (1918). He showed that this substance was removed from sensitized blood cells by an isotonic solution of sucrose, glucose, or lactose. Furthermore, it was found possible to sensitize the red cells with amboceptor, and by washing thoroughly, remove most of the amboceptor from the cells. In this way a highly purified amboceptor was secured. Apparently this has not been attempted with bacterial agglutinins, but it is entirely possible that they might be secured by a similar procedure. If so, it would constitute an excellent method for securing material for a more accurate study of their characteristics.

It has already been emphasized that the principal action of the adsorbed agglutinin is to render the sensitized cell flocculable by electrolytes. This was clearly shown first by Bordet (1899) and verified by Joos (1904) and many other investigators. This fact will be discussed more at length under the heading of electrolyte adsorption.

b Colloids which influence adsorption of other colloids. In some cases, the result of adsorption of specific agglutinin is decidedly more complex. It has been shown, for example, that adsorption of agglutinins may lead to greatly increased adsorption of certain non-specific serum proteins which in turn increase the adsorption of ions. The specific agglutinins sensitize to the serum protein, and this to the ions. Dean (1912) prepared so-called "complement fractions" from guinea pig serum by the method of Liefmann, and demonstrated that the action of anti-sheep (red blood corpuscles) sera or anti-typhoid sera were greatly increased in potency by the globulin (mid-piece) fraction.

Still more complex apparently is the phenomenon of conglutination. In this case the adsorption of the specific agglutinin increases the adsorption of a second colloid of ox serum, the conglutinin, which in turn sensitizes to a third colloid, complement, which is adsorbed and apparently sensitizes to the action of electrolytes. Muir and Browning (1906) found that the ability of anti-ox corpuscle serum from the rabbit to agglutinate ox red blood cells was greatly increased by fresh ox serum. Bordet and Gay (1906) also noted the marked agglutinative power of ox serum for sensitized red cells. This property was lost by heating the ox serum to 56°, but was restored by the addition of the complement. Bordet concluded that three distinct factors are involved; the action on the corpuscles of first, the specific serum, second the ox colloid, and third the fresh serum or alexin. Bordet and Streng (1909) named this ox serum constituent, conglutinin, and concluded that it did not need to be fixed on the cells that were conglutinated. Streng (1909) extended conglutination studies to bacteria. He found that by the use of ox serum and complement he could secure marked agglutination of bacteria in a dilution of the homologous antiserum by itself too weak to produce any trace of agglutination. When dialyzed, the agglutinin was precipitated with the globulin. Rankine (1910) also separated agglutinin and conglutinin. Hirvisalo (1913) noted that so-called exudate bacteria were vigorously clumped by normal cattle serum which had been deprived of normal agglutinins by previous treatment. This does not occur with the "native" typhoid; and evidently involves a conglutinin reaction.

The phenomenon of conglutination should not be confused with the co-agglutination of Bordet and Gengou (1911). These authors found that when a mixture of antigen and homologous antibody are added to a suspension of guinea-pig corpuscles, the latter are agglutinated. The sera used were heated to 56°C. to destroy the alexin. This reaction was obtained in all the antigen-antibody systems used, though it occurred only if a considerable excess of antigen was present and was best shown when the corpuscles were mixed with the antigen and the antibody then added.

These tests evidently show that corpuscles may adsorb specific substances from cultures which render them susceptible to agglutination.

c. Colloids which desensitize. The adsorption of a colloid may render the suspensoid less sensitive to various ions, that is, it may tend to desensitize. Bechhold (1904) for example showed that gelatin added to mastic suspension inhibited agglutination. From this he concluded that bacteria behave like suspensions possessing an albumenlike envelope, which protects the bacteria from flocculation with the salts of alkaline metals, for he found that the filtrate from twelve-day typhoid culture inhibited mastic flocculation. However, neither sensitized nor unsensitized cells changed their agglutinability in the presence of gelatin. Porges and Prantschaff (1906) concluded that spontaneously agglutinating bacteria lack the suspending action of proteins. Tulloch (1914) used 10 per cent alcohol and 1 per cent gelatin to determine the effect of differences in intrinsic pressure and internal friction on sensitized bacteria, but concluded that they were without influence.

3. Adsorption of non-electrolytes

The adsorption of non-electrolytes (not colloidal) may in some cases bring about agglutination. Malvoz (1897) found that suspensions of typhoid bacilli in distilled water were agglutinated by formalin, hydrogen peroxide and strong alcohol, but not with chloroform or phenol. He concluded that specific differences could be demonstrated between *B. typhosus* and *B. coli* by formaldehyde agglutination. His students, Lambert and Bossart (1898), could find no chemical substance that like cholera serum agglutinated specifically. Remy (1900) concluded the Malvoz formaldehyde agglutination was not strictly specific. A review of these articles seems to lead to the conclusion that in many of these instances the immediate action of the non-electrolyte is a denaturation. Gelatin, for example, is changed from a hydrophilic colloid to a suspensoid, easily flocculated, by the addition of formaldehyde. Apparently studies of bacteria

free from salts have not been made to determine these inter-relationships. In this connection it is of interest to note that in certain types of chemical reaction Bayliss (1911) has found adsorption to be an essential preliminary.

4. Adsorption of electrolytes

a. Hydrogens ions. Most important as the apparent immediate cause of agglutination is the adsorption of cations by the bacteria. Studies thus far made show little effect of various anions, though that they are entirely without influence is scarcely probable. However, the influence may be disregarded for purposes of a general discussion.

We may, for convenience, discuss this effect under the heading of concentrations of hydrogen ions, and of ions of univalent, divalent, trivalent and polyvalent bases.

Bechhold (1904) noted that acids are very active in producing agglutination, the most powerful being those most strongly dissociated, such as HCl. The weak amidobenzoic acid flocculates poorly, while acetic acid is intermediate in its action. He found that M/1000 HCl or H₂SO₄ agglutinated unsensitized and M/2000 sensitized bacteria. With ortho-amido-benzoic acid 5M/1000 was required for both sensitized and unsensitized cells. Michaelis (1911) found that in a series of solutions with varying hydrogen ion concentration but constant salt content he secured flocculation with *B. typhosus*, at a hydrogen ion concentration of 4×10^{-5} N. Beniasch extended these observations to a large number of bacteria. For *B. typhosus* he found maximum flocculation at $P_{\text{H}} = 4.45$, though some variation with different strains was detected. In the *B. enteritidis* series two subgroups were described with optima at $P_{\text{H}} = 3.86$ and $P_{\text{H}} = 2.66$. With *B. coli* and *B. dysenteriae* he could detect no acid agglutination between $P_{\text{H}} = 7$ and $P_{\text{H}} = 2$. He concluded that the acid agglutinable substance of the bacterium is identical with the substance agglutinated by the specific serum. These findings of Michaelis and Beniasch have been verified by many subsequent studies. Michaelis and Davidsohn (1912) concluded that specific

serum agglutination could not be explained upon the basis of affinity due to electric charges. They found that the more concentrated the agglutinin, the less was the agglutination dependent upon the hydrogen ion concentration. Ostwald (1913) and Landsteiner (1913) urge that Michaelis and Davidsohn (1911) do not prove their point and have not invalidated the colloidal theory of immunity reactions. Krumwiede and Pratt (1912) also note that specific serum agglutination occurs over a wide range of P_{H} values. They found, however, that sensitized bacteria would agglutinate in tube 1 of a Michaelis series and to a much higher serum dilution than with salt solution. McIntosh and McQueen (1914) showed that typhoid bacilli agglutinable with serum as well as non-agglutinable strains were both clumped by a hydrogen ion concentration of 3.5×10^{-5} to 8×10^{-5} N. They contend that this does not support the view of Beniasch that agglutination by acid and serum depend upon the same factors.

Gillespie (1914) used one of the Michaelis series with pneumococci, and found decided salt interference with agglutination by acid in some cases. Young cultures of type I gave a narrow zone of agglutination with optimum hydrogen ion concentration of 5.5 to 11×10^{-4} N; ten strains of type II reacted to 22×10^{-4} . Ten strains of atypical pneumococci showed great variation. The atypical forms were uniformly lacking in salt susceptibility. Attention may be again called to table I, showing the range of acid agglutination with the meningococcus. In summary, it may be stated that although numerous attempts have been made to use acid agglutination as a specific test for the differentiation and recognition of bacteria, the method has apparently not found favor, and has not replaced the use of specific antisera.

b. Univalent cations. Bechhold (1904) regards univalent ions of the alkaline metals as of little significance in the agglutination of unsensitized bacteria. Mastic suspension, which is more sensitive, he found flocculated by high concentrations, as for example, M/1 NaCl. Typhoid cells, sensitized and washed, were flocculated by 0.025 N NaCl. Similar results were

secured by Neisser and Friedemann (1904). Crenderopoulo and Amos (1904) showed evidence of antagonistic action of sodium and potassium salts. Eisenberg and Volk (190-) studied the effect of various salts on agglutination of sensitized cells, and concluded that sodium chlorid favored, while potassium chlorid inhibited such agglutination. Porges and Prantschoff (1906) dialyzed spontaneously agglutinable typhoid organisms, and found that this treatment somewhat decreased their agglutinability with salts. A trace of agglutination was secured with N/8 NaCl and complete agglutination with N/2 NaCl. KCl, KNO₃ and NaNO₃ all required equivalent concentrations. Porges (1906) tested unsensitized cholera bacteria with (NH₄) SO₄ and secured partial agglutination with 20 and 30 per cent saturation and complete with 40 per cent. Typhoid bacilli showed partial agglutination at 40 per cent saturation and complete agglutination at 50 per cent; *B. coli* gave partial agglutination at 40 per cent and complete agglutination at 60 per cent. Friedlander's bacillus was agglutinated partially at 60 per cent and completely at 70 per cent. It may be noted that about the same series would be secured by rating these bacteria on the basis of ease of agglutination with specific sera.

Verzar (1917) studied the reaction of a typhoid strain showing so-called spontaneous agglutination in physiological salt solution. He found that by diluting the salt solution to one half or one quarter its strength, he no longer secured spontaneous agglutination but could get specific agglutination with antisera.

Porges (1906) mixed dialyzed bacteria and dialyzed antiserum 1-100 and determined the concentration threshold of numerous cations necessary for flocculation. With NaCl, KCl, KNO₃, NH₄NO₃ it was M/500, with Na₂SO₄ M/1000, K₂SO₄ M/1500, (NH₄)₂SO₄ M/1200. It will be noted that apparently the anion is without effect, and the cations Na, K and NH₄ are about equipotent.

Tulloch (1914) studied the effect of the electrolyte upon the titre of a serum. He tried NaCl, NaF, Na₂SO₄, K₂SO₄, Na₂HPO₄, BaCl₂ and Na₂Cit, equimolecular with 0.9 per cent NaCl; all caused agglutination with dilution of 1-8000 of serum, and

not 1-16,000. Salts which hydrolyze to produce OH ions do not agglutinate. Chart I gives the results secured with various concentrations of antimeningococcic serum, sodium chlorid and

TABLE 4

Agglutination of meningococcus cells sensitized with immune serum and washed. Antigen no. 60. Turbidity 500 parts per million. Incubated at 56°C. twenty hours

NaCl	AGGLUTINATION	CaCl ₂	AGGLUTINATION	Ce ₂ (NO ₃) ₃	AGGLUTINATION	SERUM	AGGLUTINATION
M/2	3	M/2	4	M/20	4	1/4	4
M/4	3	M/4	4	M/40	?	1/8	3
M/8	3	M/8	4	M/80	0	1/16	1
M/16	4	M/16	4	M/160	0	1/32	0
M/32	4	M/32	4	M/320	0	1/64	0
M/64	3	M/64	4	M/640	0	1/128	0
M/128	3	M/128	4	M/1280	0	1/256	0
M/256	0	M/256	4	M/2560	0	1/512	0
M/512	0	M/512	3	M/5120	3	1/1024	0
M/1024	0	M/1024	2	M/10240	4	1/2048	0
M/2048	0	M/2048	0	M/20480	1	1/4096	0
M/4096	0	M/4096	0	M/40960	0	1/8192	0

TABLE 5

Agglutination of meningococci with ammonium sulfate. Comparison of sensitized and nonsensitized cells. Strain no 55.

CONCENTRATION (NH ₄) ₂ SO ₄	SENSITIZED CELLS	UNSENSITIZED CELLS
2M	4	4
M/1	4	4
M/2	3	2
M/4	3	1
M/8	3	1
M/16	4	0
M/32	4	0
M/64	4	0
M/128	4	0
M/256	2	0
M/512	0	0
M/1024	0	0

meningococcus antigen. It is evident that an excess of sodium chlorid inhibits the specific agglutination. The explanation of the form assumed by the area showing complete agglutination

is apparently not a simple one. Strangely enough in certain concentrations of NaCl a decrease in the concentration of the serum increases the agglutination.

Table 4 shows the behavior of sensitized washed meningococci in the presence of different dilutions of various salts.

Table 5 shows the agglutination of both sensitized and unsensitized washed meningococci with ammonium sulphate.

3. Divalent cations

Bechhold (1904) found divalent bases stronger as agglutinating agencies than univalent ones. The salts of the alkaline earths such as barium chloride acted on mastic but not on normal bacteria, sensitized bacteria were flocculated in a dilution of 5 M/1000. Zinc sulfate and zinc nitrate agglutinated sensitized bacteria to a dilution of M/1000 and unsensitized bacteria to M/100. Other divalent metals showed high agglutinating power, varying from M/10,000 with sensitized to M/400 with unsensitized cells. Malvoz (1897) secured agglutination of typhoid bacilli in distilled water with 0.7 parts per thousand of mercuric chlorid. Crenderopoulo and Amos (1904) found that sensitized cholera vibrios agglutinated well with CaCl_2 1 part to 10,000, this being five times as dilute as the effective solution of NaCl. Porges (1906) calculated that magnesium ions were about ten times as potent as univalent ions in producing flocculation of sensitized typhoid cells, the threshold values being for MgCl_2 M/7500, for $\text{Mg}(\text{NO}_3)_2$ M/5000 and for MgSO_4 M/10,000. He argues that inasmuch as magnesium salts will not salt out this organism from normal suspensions, the mechanism must be different with sensitized bacteria.

Liefmann (1913) attempted to find specific differences among organisms by "salting out," particularly with MgSO_4 . Paratyphoid bacilli of the Gärtner type he found to be flocculated by 80 per cent saturation, but neither *B. typhosus* nor *B. coli*; the latter form was usually not salted out even with 90 per cent concentrations. I have found no record of agglutination of unsensitized bacteria by salts of calcium. I was therefore sur-

prised to secure with meningococcic antigen the results given in table 2. It will be noted that with some strains a zone of complete agglutination at about M/64 is manifest. Either excess or deficiency of calcium ions may apparently inhibit flocculation. It is possible that other bacteria will show similar results upon further study.

4. Trivalent bases

Bechhold (1904) found the trivalent bases to be particularly powerful agglutinants. Iron and aluminium sulfate were found to flocculate in dilutions of M/10,000, five times as dilute as a solution of HCl giving similar agglutination. Bechhold secured a very good example of an irregular series with both sensitized and unsensitized bacteria and $\text{Fe}_2(\text{SO}_4)_3$, and also with sensitized bacteria and PtCl_4 . These irregular series were observed only with trivalent salts that showed strong hydrolysis, the colloidal iron oxid in the inhibition zone apparently preventing the flocculation. Neisser and Friedemann (1904) showed that when a cation shows a variable valence in its salts, its agglutinative power is in accordance with the higher valence. Burton and Rahe (1909) also emphasize that with salts of the trivalent metals, on account of hydrolytic dissociation there is a combination of the electrolyte type and colloidal type of flocculation giving rise to irregular series.

Table 3 illustrates the mixed agglutination series secured with meningococci and cerous nitrate.

5. Polyvalent bases

Bechhold (1904) used PtCl_4 finding its threshold at about five times the concentration of iron. Tulloch (1914) found that TaCl_5 gave a zonal agglutination. In some of my own work I have found that iridium chloride, IrCl_3 , gives a regular agglutination series, and in relatively high dilutions.

Perhaps in a distinct category should be noted the precipitating power of certain organic salts particularly salts of the alkaloids. Sabrazes and Brenques (1899) noted that salts of quinine and atropine cause agglutination.

6. *Relation of valence to power of agglutination*

Bechhold (1904) first emphasized that with bacteria as with proteins, the power of a cation to cause flocculation increases with the valence. The same observation has been made by many subsequent writers. One of the best demonstrations of this fact is that of Tulloch (1904) who found that with sensitized cells NaCl and KCl gave agglutination to 0.002 N, while CaCl_2 , StCl_2 , BaCl_2 and MgCl_2 were all active up to 0.0004 N. He agrees with previous writers that the agglutinating activity of an electrolyte depends on the valency of the cation. He compared the effect of NaCl, BaCl_2 and LaCl_3 on sensitized cells with the following results. The highest dilution of the salt showing agglutination is given.

Sensitized.....	NaCl 0.004 N	BaCl_2 0.0004	LaCl_3 0.00003
Unsensitized.....	NaCl 0.004 N	BaCl_2 0.0004	LaCl_3 0.00003

Tulloch concluded that the law of increased activity of cations with increase in valence, $1 : x : x^2$ as suggested by Linder and Picton (1895) held at least, approximately. Salts with polyvalent cations behave differently from those of mono and divalent cations. Apparently they are as active in the agglutination of unsensitized as sensitized bacteria.

7. *Denaturation or sensitization by non-colloids*

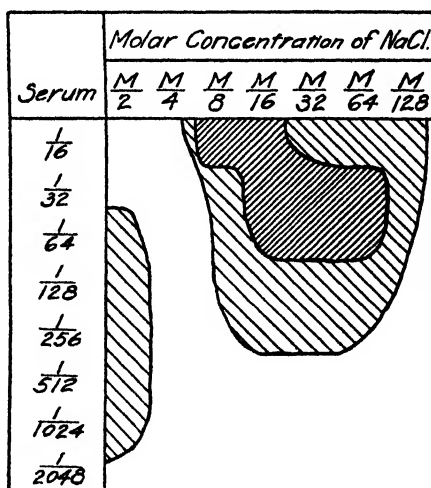
Several instances are on record of the sensitization of bacteria to the action of an electrolyte by the action of a non-electrolyte. For example, Bechhold (1904) found that bacteria treated with alcohol and washed were agglutinated by strong NaCl, and by even relatively weak dilutions of divalent cations. The same author also showed that bacteria could be sensitized by contact with lead acetate or uranyl acetate followed by washing. Imai (1912) studied the effect of osmium on bacterial agglutination and found the osmated bacteria more strongly agglutinated than normal.

Tulloch (1914) sensitized bacteria with aluminium and studied the flocculation with various salts. The agglutination thresh-

hold of the sensitized bacteria for the various salts were as follows: NaCl N/200; BaCl₂ N/2000; LaCl₃ N/5000; Ce₂Cl₆ N/10,000; AlCl₃ N/500 negative zone and N/1000 to N/10,000 positive. With unsensitized cells the results were as follows: NaCl, all negative; BaCl₂, all negative; LaCl₃ N/5000; Ce₂Cl₆ N/20,000; AlCl₃ N/1000 negative and N/2000 positive; N/20,000 positive and N/40,000 negative.

8. Graphic representation of action of mixtures

Probably one of the fertile fields for investigation will prove to be a study of the effect of varying two or more of the constituents of the mixture which under certain conditions may cause



Legend:


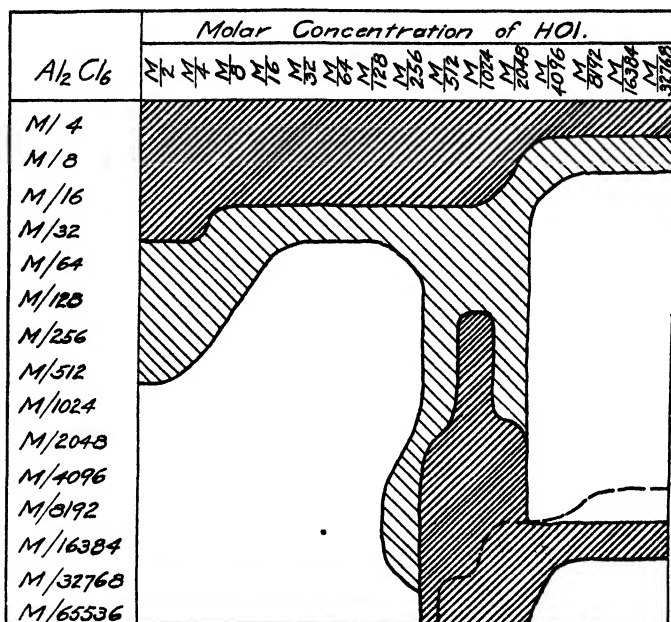
-  Complete Agglutination
-  Partial " "
-  No " "

CHART I. AGGLUTINATION OF MENINGOCOCCUS BY VARIOUS MIXTURES OF IMMUNE SERUM AND SODIUM CHLORID

Antigen no. 60. Turbidity 500 parts per million. Incubated at 56°C. for twenty hours.

agglutination. This has been done for typhoid bacteria, anti-typhoid sera and various electrolytes by several writers.

Porges (1906) dialyzed typhoid bacteria and the homologous antiserum until chlorin free. With serum in dilution of 1-10



Legend.

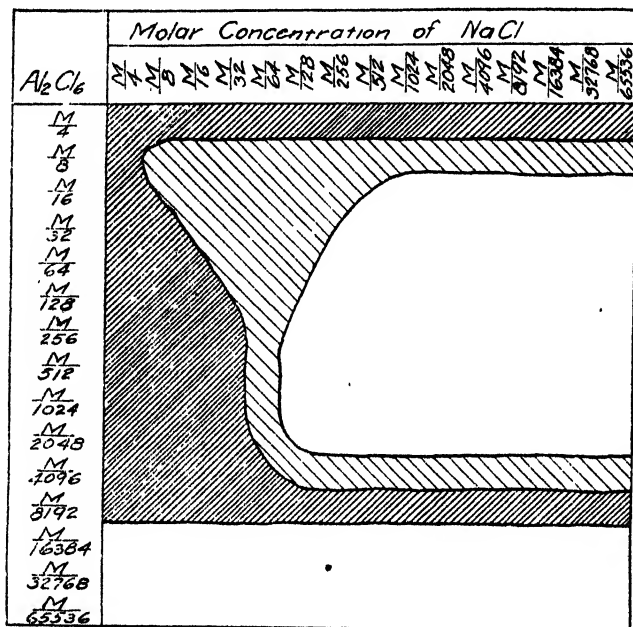
- Complete Agglutination
 Partial "
 No "

CHART II. AGGLUTINATION OF MENINGOCOCCUS BY VARIOUS MIXTURES OF ALUMINIUM CHLORID AND HYDROCHLORIC ACID

Antigen no. 55. Turbidity 500 parts per million. Incubated at 56°C. for twenty hours.

he secured agglutination without NaCl, but not in any higher dilution. N/500 NaCl gave partial agglutination with a dilution of 1-500 of serum and N/50 NaCl, partial agglutination with 1/5000 dilution of serum. The general rule deduced was

that with increasing amounts of agglutinin the amount of salt necessary to agglutination decreases. Tulloch (1914) studied the relationships between concentration of serum and of elec-



Legend:




-  Complete Agglutination.
-  Partial " "
-  No " "

CHART III. AGGLUTINATION OF MENINGOCOCCUS BY VARIOUS MIXTURES OF ALUMINIUM CHLORID AND SODIUM CHLORID

Antigen no. 59. Turbidity 500 parts per million. Incubated at 56°C. for twenty hours.

trolites and came to similar conclusions. He used both NaCl and BaCl₂.

Walpole (1914) has introduced a valuable diagrammatic method of studying the effect of varying the concentration of two different substances on the flocculation of a sol, which he applied to

the effect of varying gelatin and acid concentration upon the flocculation of an oil emulsion. An understanding of the effects of various combinations upon bacteria will be facilitated by pursuit of a similar method.

Chart I illustrates this method as applied to variable anti-serum and variable sodium chlorid concentrations on meningococcic antigen.

Chart II plots the agglutination of a meningococcus antigen by varying proportions of aluminium chlorid and hydrochloric acid. This chart shows zones of agglutination by hydrogen ions, by aluminium ions, and by the aluminium hydrate, and zones of increased agglutination due to the combined action of hydrogen and aluminium ions. Areas are also shown in which there is inhibition by the aluminium of acid agglutination, and others in which the acid inhibits the action of the aluminium.

Chart III illustrates similar facts for various mixtures of aluminium chlorid and sodium chlorid.

CONCLUSIONS

There is ample justification for the conclusion that bacterial agglutination is a colloidal phenomenon that can best be studied in the light of the modern work on colloidal and physical chemistry. There is real need for intensive study of the complex inter-relationships of the factors which govern this phenomenon that they may be more clearly understood, and the diagnostic use of agglutination put upon a more scientific, rather than a purely empirical basis.

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METHODS OF PURE CULTURE STUDY

PROGRESS REPORT FOR 1918 OF THE COMMITTEE ON THE DESCRIPTIVE CHART OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

H. J. CONN, *Chairman*, H. A. HARDING, I. J. KLIGLER, W. D. FROST,
M. J. PRUCHA, AND K. N. ATKINS

Received for publication October 10, 1918

I. THE DESCRIPTIVE CHART AND ITS PURPOSE

Since 1905 there has been in existence, with occasional modification, a chart endorsed by the Society of American Bacteriologists for recording the characteristics of bacterial cultures. Recent literature, however, indicates that some confusion exists as to the purpose of this chart. This confusion was well illustrated at the 1917 meeting of the Society, the discussion called forth by the report of this committee showing that a number of bacteriologists have ideas as to the object of the chart different from those held by the present members of the committee. The confusion is natural, for each change in the membership of the committee has caused the chart to develop along slightly new lines; and even the early members of the committee have gradually changed their ideas as the result of later experience. It seems desirable, therefore, to restate the purpose of the descriptive chart.

The first bacteriologists to devise descriptive charts did so on individual initiative and apparently had in mind merely the concise description of their cultures preliminary to publication. An examination of the cards of Gage and of H. W. Conn¹ suggests that nothing further than this was expected of these two charts. Later, however, it was felt that uniformity in the cards used by

¹ For a description of these cards the reader is referred to a paper by H. A. Harding (1910).

different workers would make it possible to compare the results obtained in different laboratories. The Society, therefore, appointed a committee to prepare an official chart, and in its report this committee expressed itself as follows:

The past literature of bacteriology abounds in such imperfect descriptions of organisms as to make their grouping, according to any system, impossible. This fact calls for the adoption of some scheme to which all descriptions shall conform, in order that no essential character shall be overlooked. The accompanying card is proposed for the recording of the characters of an organism. . . . The cards can be filed like catalog cards, and arranged in accordance with the group number, thus bringing similar organisms together and rendering comparison easy.

This seems to imply that the chief objects intended to be served by the chart are first to secure uniformity in published descriptions and second to facilitate the grouping of bacteria according to their characteristics. Recent literature, however, reveals the existence of an idea not expressed in this report: namely, that the group number on this official card furnishes a more satisfactory method of characterizing an organism than its identification with a specific name occurring in the literature. The idea probably had its origin in the form of group number introduced with the Society card. Gage and Conn had both used a group number, but their group number consisted merely of four digits which could have suggested to no one that it constituted a complete description of a species. The Society group number, however, was simplified by leaving off those digits that indicated form and motility, replacing them with the genus symbol (according to Migula), which was placed before the series of digits. That this use of the genus symbol was not considered by the first committee to convert the group number into a method of characterising species is shown by the following statement which they made in regard to the group number:

This system readily enables organisms having similar characters to be brought together and grouped about some central organism or type.

The only hint in this report that the group number might replace a specific name occurs a little later when the statement is made that according to this method of expression "*Bacillus coli* (Escherich) Migula becomes B.212.33310." This statement suggests to the reader, especially if that reader be a beginning student in bacteriology, that the string of digits 212.33310 is equivalent to the specific term *coli*. It naturally led to the impression on the part of the part of certain younger bacteriologists that the group number is nearly all-sufficient in describing a new species.

This point of view was strengthened by a paper from a member of this committee (Harding, 1910) on *Ps. campestris*, showing this organism apparently to have a constant group number. This work did not show that no other organism has the same group number, nor that all other organisms are so constant in physiological characteristics as is this particular species, nor indeed that this organism would have given such constant results if the technic had been varied; but there has been some tendency on the part of other bacteriologists to read into the paper a conclusion of this nature. The statement has even been made in one recent paper that the group number obviates the necessity of bacterial names.

On the other hand, various investigators, using other species of bacteria, have one by one criticised every point in the group number except that which refers to spore-production, showing that the same culture may give different results each time the tests are repeated. Further criticisms have arisen from the biometric studies of bacteria, such variations having been observed among the different strains of a single species that no characterization of a species can be considered valid until the mean is determined around which the different strains vary. These criticisms have sometimes been met by the statement that if the technic were sufficiently perfected and standardized, each strain would always prove to have the same group number, even though that group number might not express the characteristics of the species under all other possible conditions. An objection to this argument has been pointed out by Breed (1914)

in his work on the nitrate reduction of *B. coli*. He shows that the use of a standard medium, instead of distinguishing sharply between organisms with positive reactions and those with negative reactions, is likely to bring about variable results for those which grow poorly in the medium adopted. Recent work of the committee, moreover, shows very plainly that (in regard to nitrate reduction and acid production at least) no standard medium can be adopted which will give consistent results with all kinds of bacteria.

In brief, then, it may be said that the descriptive chart provides a uniform and concise method of recording nearly all of the ordinary and some of the unusual observations concerning the morphology and activities of an organism. Of the many characteristics listed on the chart, about a dozen were selected by former members of the committee as being the most important and were collected into a numerical expression called the group number. This group number is merely an abbreviated method of recording these characteristics, and must not be regarded as a sole means of characterizing species. Its value as a means of characterizing cultures, or strains, or species depends not only upon the wisdom with which these particular reactions have been selected but also upon the accuracy with which each individual characteristic can be determined. In both of these respects any group number proposed at present would necessarily be imperfect: first, because the diagnostic importance of the various characteristics differs among different groups of bacteria; and secondly because the methods for making the determinations have never been perfected and the results are correspondingly inaccurate. The problems of the committee, therefore, include the selection of the characteristics of greatest diagnostic value and the elaboration of methods which shall minimize mistakes. The committee is now studying the methods for working out the old group number; but, as a more permanent improvement, a new group number is being planned, less arbitrary and more logical than the one now on the card.

In addition to these weaknesses arising from our imperfect knowledge of bacterial activities and the methods of determin-

ing them, the card has not been revised frequently enough to contain the more recently devised tests for special groups of bacteria. Hence it is coming to be less and less used as a means of publishing descriptions of bacteria. In other words, it no longer helps to secure uniformity in bacterial characterization, the purpose for which it was originally intended.

Meanwhile instructors in bacteriology have realized the advantages of some such method of concise description in familiarizing students with the characteristics of bacteria. Some have used the official descriptive chart; but this chart was never intended for instruction purposes and is not designed to be put in the hands of beginning students. As a result other instructors have prepared descriptive charts of their own. Each of these charts has its own advantages and is undoubtedly better adapted to instruction than the old Society card; but for obvious reasons it would be better if a uniform style of chart were used in all institutions. The present committee, therefore, was instructed to prepare a chart especially adapted to use in bacteriological instruction: A new folder was prepared in response to these instructions and was presented at the 1917 meeting. It is now in print and is available to any teacher who wishes to try it out in his classes.

As the descriptive chart is now chiefly used for instructional purposes, it seems wisest to emphasize this use of it; but the committee does not wish to imply that an improved form of chart would be of no use in research work. The society card, indeed, has proved well adapted to certain types of research work in which a general survey is desired of the bacterial flora of some particular medium, preliminary to a later, more intensive, study of the individual species.² For some of this work the newer chart, although intended primarily for instruction, may prove more useful than the old form; but the old card is still available for use in this type of work whenever it is desired. Better still, for this purpose, would be a new card more nearly in accord with modern methods of characterizing bacteria, and

² Preliminary flora studies of this nature are well illustrated by the work of Conn, Esten and Stocking (1906) and by that of Harding and Prucha (1908).

specially adapted to research work. The committee hopes to prepare such a card in the future; but before that is done more attention must be given to the value of the various characteristics and to the methods of determining them.

The 1917 report of this committee (Conn. et al. 1918) contained a discussion of methods of pure culture study, designed primarily to accompany the chart for instruction. Further work has been done on some of these methods during the past year. A year ago it was expressed as the desire of the committee that these methods "be adopted as standard methods at the 1918 meeting, after such changes have been made in them as the year's use shows to be necessary." This is no longer urged. With the modifications given in the following pages, they are the best methods now known to the committee, and their use in instruction laboratories is strongly recommended; but as the results of criticisms of the 1917 report, together with the committee's further work during 1918, it seems wisest to regard them as provisional methods. No action is therefore asked on these methods and the committee desires to do further work on them.

The following pages contain a progress report on those methods that have been investigated during the past year, namely: Gram stain, acid production, and nitrate reduction. Free criticism of all matters discussed in this report, as well as the methods outlined in the preceding report, will always be welcome.

II. THE GRAM STAIN

At the last meeting of the Society one or two members criticised the classification of bacteria according to their ability to take the Gram stain, stating that many of the methods of making this test give irregular results and that the methods given in text books vary greatly from one another. The committee was asked to look into the matter. Gram originally gave no definite length of time for treatment in the different fluids; but as the results of the staining depend greatly upon the duration of each treatment, various investigators have felt it necessary to specify

definite periods of time. Unfortunately, however, scarcely any two investigators have agreed in the periods specified.

From two of the members who particularly objected to the lack of agreement between present methods a statement has been obtained as to the technic giving best results in their laboratories. The two methods thus obtained differ considerably: one method calls for a comparatively concentrated stain, a short period of staining and long decolorization, the other for a weaker stain, longer staining period and short decolorization. The former method is less time-consuming and the gentian violet solution it is claimed deteriorates more slowly; but upon comparison of the two methods no further differences have yet been observed. Both methods have been found to give clear-cut distinctions between the Gram-negative and Gram-positive organisms thus far studied. The committee realizes the advisability of adopting a standard technic, but feels that no method should be made standard without more investigation than has so far proved possible. Both methods are therefore given as capable of yielding good results; and the criticism of the Society upon them is invited.

Method 1

Gentian violet solution.....	1 minute
(Stain prepared by grinding 5 grams in 10 cc. of 95 per cent alcohol in a mortar. Add 2 cc. anilin oil, distilled water 88 cc. Filter. Claimed to keep 3 to 4 months.)	
Iodine solution (Lüggol's).....	1 minute
(As usual: Iodine 1 gram, potassium iodide 2 grams, water 300 cc.)	
Absolute alcohol.....	2 minutes
Counter-stain.....	30 seconds
(10 cc. of saturated alcoholic safranin in 90 cc. water.)	

Method 2

Gentian violet solution.....	3 minutes
(Anilin oil 3 cc., absolute alcohol 7 cc., water 90 cc. Shake and filter through moist filter. Add 2 grams of gentian violet and allow to stand 24 hours.)	
Iodine solution (Lüggol's, as above).....	2 minutes
Absolute alcohol.....	30 seconds

Counter-stain:

Either Fuchsin (one part saturated alcoholic solution to nine parts of water)	30 seconds
or Bismark brown (2 per cent, dissolved in hot water and filtered)	2 minutes

Whichever method is used there are certain points in the technic that are important and must be insisted upon, especially if the test is being made by a beginner. The medium used must be recorded. Only young cultures should be used (eighteen to twenty-four hours old, except in case of unusually slowly growing organisms). Films should be on cover slips, made with distilled water, and in the portions examined organisms should be only one layer thick. Gentian violet and iodine solutions should each be removed by merely draining off the excess and blotting, before applying the next solution. Cover slips should be kept in constant agitation while in the alcohol and should then be transferred without washing to the counter-stain.

III. THE PRODUCTION OF ACID

Four places in the group number are devoted to the fermentation of sugars and glycerin. In the 1917 report the committee recommended studying this reaction in standard peptone broth. There are various possibilities of error inherent in this method, and it is not applicable to all bacteria. Accordingly a study has been made of the sources of error and the simplest methods of overcoming them.

Sources of error in determining acid production

One of the chief sources of error in determining acid-production comes from the weakness of the common methods of detecting and measuring acid. A change in the titre of any medium as the result of bacterial growth is not necessarily in the same direction as the actual change in H-ion concentration. This makes the use of the titration method unsatisfactory for detecting acid production. This source of error, however, may be overcome by the use of the proper indicators if the student has

a good comprehension of the principles of H-ion concentration. A simple but satisfactory method of using these indicators and interpreting the results was given in the 1917 report (pp. 122-124). The significance of these indicators and their use is discussed in more detail in the following pages.

Certain other sources of error are not so readily eliminated. Among them is the use of media in which the organisms in question make poor growth. If an organism does not grow well in a medium it is not correct to state that it is unable to produce acid from the sugar present in that medium. Certain organisms do not grow well in ordinary broth because they require more nitrogenous matter and a suitable medium for them should be found before adding the carbohydrate upon which their action is to be studied. Others require a different initial reaction from that of ordinary standard media. Others grow well only on solid media and give consistently negative results if tested in liquid media, merely because of their poor growth.

Another equally serious source of error is the fact that many bacteria may cause two simultaneous biochemical activities, one tending to raise the reaction, the other to lower it. The resultant change in reaction depends upon which of these two processes predominates, and the production of acid may often be entirely masked by the opposite tendency. This may often be the cause of variation in the results obtained upon repetition of the test with the same culture; and it may even cause an acid-producer to escape recognition entirely.

Error may also arise from impurities in the sugars used. Glucose is very often present as an impurity in other sugars, especially in those which are difficult to purify, but has also been observed in samples of sucrose and lactose. The presence of glucose can be detected by growing in it a known glucose fermenter that does not attack the sugar in question. This test should be made in all accurate work.

Media recommended

On account of these difficulties, it proves impossible to recommend any one standard medium. The formulae recommended

(J. Bact., 3, 115, 116) are merely for routine use when the growth requirements of an organism are not known. If the organism grows poorly in these media, the results of the test are to be disregarded.

In connection with preliminary invigoration, the committee has already recommended (J. Bact. 3, 118) dividing the organisms into four series according to their preference for 37° or 25°, and for liquid or for solid media. It is now further recommended that all organisms of series III and IV (i.e., growing well on the surface of agar but not in glucose broth) be tested for acid production by inoculating onto the surface of beef-extract agar (instead of broth) containing the carbonaceous substance to be tested. Bacteria not growing well in glucose broth or in beef extract agar must be tested in some medium in which they do make good growth. For them is recommended varying first the peptone content and then the acidity of the medium. The medium to which the sugar, glycerine or other substance is added should differ as little as possible from these standard media.

Methods of detecting acid production

For the reasons above discussed acid production should not be detected by means of titration. An indicator should be used whose range of color change covers the initial reaction of the medium used or else is slightly to the acid side of the initial reaction. (Thus brom cresol purple, with the range of $P_H = 5.2$ to $P_H = 6.8$ is the most satisfactory indicator to use with media of initial reaction of $P_H = 7.0$) Litmus can be used, but the results obtained with it are not sufficiently clear cut.

Indicator media are especially valuable now that we have indicators which are not attacked by bacteria, with turning points at the useful part of the P_H -scale. Litmus and methyl red are of but little use for this purpose because they are decolorized by bacteria; but the sulphone-phthalein indicators are not acted upon by bacteria and are just as satisfactory when mixed with the media before inoculation as afterward. The

addition of the minute quantities of these indicators necessary does not ordinarily influence the growth of the bacteria, and in most cases its influence can be disregarded.

The use of saccharine broth or agar containing brom cresol purple gives very clear indication as to acid production from the sugar present, unless the organism produces alkalinity from the peptone. This possibility should always be investigated by the use of some indicator with a more alkaline range, such as phenol red or cresol red. In case alkalinity is produced in the broth or agar without the sugar, it must be recognized that the production of acid may not raise the H-ion concentration of the medium above its original reaction. Theoretically the only way to be sure of acid production in such cases is to use some medium in which the organism grows well without producing alkalinity; but as such a medium is ordinarily difficult or impossible to obtain, this procedure is generally out of the question. The method recommended is to use a non-saccharine broth or agar as a check, both saccharine and non-saccharine media containing those indicators whose ranges cover the P_{\pm} changes likely to occur, and to pronounce the organism an acid-producer provided the H-ion concentration is greater in the presence of the sugar than it is in its absence. A very handy combination of indicators for this test is a mixture of brom cresol purple and cresol red. They may both be added to the same medium, giving a solution that changes very slowly from purple to yellow through a long range (from about $P_{\pm} = 8.0$ to about $P_{\pm} = 5.0$) extending to a considerable distance on both sides of neutrality. The exact amount of indicator used in the medium is not important as long as the color obtained is distinct. A convenient strength has been found to be 1 cc. of a 1.6 per cent alcoholic solution to the litre of medium. When brom cresol purple and cresol red are mixed, neither indicator need to be used in quite such great concentration.

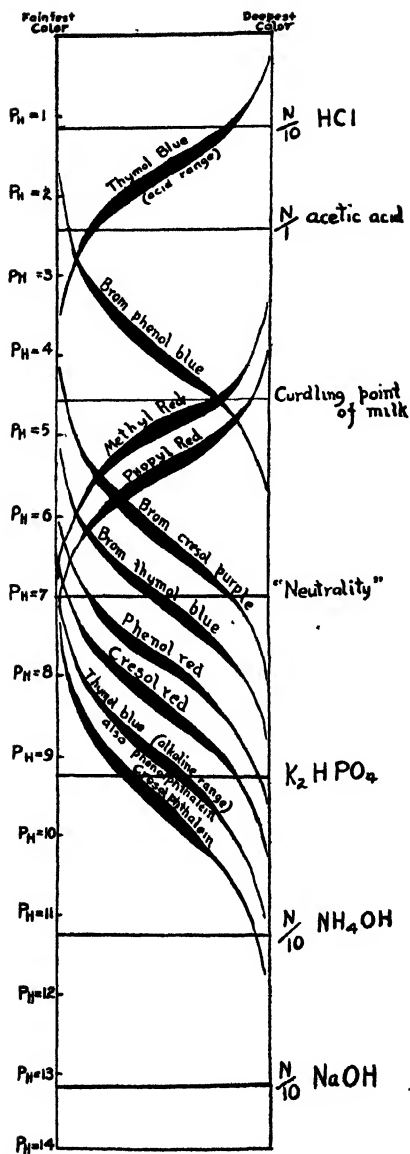


FIG. 1. COLOR CHANGES IN CLARK AND LUBS' INDICATORS. COMPARED WITH THE P_H VALUE OF CERTAIN IMPORTANT SOLUTIONS
Slightly modified from chart of Clark and Lubs

Relation of H-ion concentration to acidity

The above discussion will be readily intelligible to any reader who has an understanding of H-ion concentration and of the meaning of the symbol P_H . There would, indeed, be no point in discussing this subject further, if the research worker alone were concerned, because Clark and Lub's recent paper (1917) is readily available and treats the subject as fully and adequately as could be desired. The new chart, however, is intended primarily for instruction and it is felt that teachers may desire a brief discussion of hydrogen-ion concentration for class-room or reference use. As there is at present no bacteriological textbook, so far as the committee know, that discusses the relation of acidity and reaction of media to H-ion concentration, and as copies of this report can be obtained from the secretary of the Society at about cost price, it seems well to summarize the subject here. Nothing new, however, is added in the following pages to what previous writers have said.

What is hydrogen-ion concentration?

The concentration of hydrogen-ions in a solution is used as a measure of acidity and alkalinity on the basis of the ionic theory. According to this theory, every salt, acid, or base, in aqueous solution, breaks up to some extent into "ions." Even pure water (H_2O) dissociates to a very small extent into H-ions and OH-ions. The H-ions bear a positive electric charge, the OH-ions a negative charge. Free H-ions are acid, free OH-ions are basic. In pure water both are present in equal amounts, thus neutralizing each other. Acids and bases dissociate to a much larger extent than pure water, the amount of dissociation depending ordinarily upon their strength. An acid dissociates into positive H-ions and negative ions consisting of the acid radicle. A base dissociates into positive metal ions and into negative hydroxyl (OH) ions.

The stronger acids (sulphuric and nitric, for instance) ionize to a greater extent than the weaker (acetic, for instance, and other organic acids). The more completely an acid ionizes in

solution, the greater the H-ion concentration of that solution. Therefore if different acids are dissolved in the same ratio of acid to water the H-ion concentration is an index of the strength of the acid. Similarly if different bases are dissolved in water in the same proportions, the OH-concentration is an index of the strength of the base.

If to a quantity of pure water (containing H and OH-ions) a drop of sulphuric acid be added, the mixture will contain H-ions, OH-ions and SO_4 -ions. The number of free H-ions will be greatly increased because of the large number added in the drop of acid; but the number of hydroxyl ions will be decreased, because the change in ionic equilibrium will cause many of them to combine with free H-ions to form water. In other words, the H-ion concentration will be increased, the OH-ion concentration decreased. If, further, a larger quantity of the acid be added, the H-ion concentration will be still further increased, the OH-ion concentration still further decreased. It is evident, therefore, that H-ion increases and OH-ion concentration decreases as the acidity increases. Either may be used as an index of acid reaction; but H-ion concentration is preferred because it increases with increasing acidity.

When an acid is mixed with water containing other materials in solution, the matter is more complex. Various combinations are possible between the acid and the other materials in solution which prevent the H-ion concentration from being increased as much as it would have been if the same amount of acid had been added to pure water. Materials which thus repress the H-ion concentration of a solution are called "buffers." Organic matter in solution is especially likely to exert a buffer action. When buffers are present, the H-ion concentration of any solution will obviously be lower than it would have been with the same amount of acid added if no buffers were in solution. The actual reaction of such a solution is also lower, for only the ionized portion of an acid exhibits acid properties. The H-ion concentration, therefore, is a more correct index of the acid or alkaline reaction in a case like this than is the quantity of acid added.

When a base is added to water or to a neutral solution, the relation is exactly the reverse. The concentration of OH-ions is increased, that of H-ions is decreased. Either may be used as an index of alkalinity in terms of the ionic theory; but H-ion concentration is ordinarily used for the sake of uniformity. Hydrogen-ion concentration is inversely proportional to the dissociation of the base and therefore to the true alkaline reaction of the solution. Obviously a saturated solution of a strong base such as KOH has an exceedingly small H-ion concentration, but it is nevertheless measurable.

What is meant by P_H ?

After deciding to use H-ion concentration as a measure of reaction the first necessity is to obtain some simple and concise method of expression. For this purpose the figures actually expressing H-ion concentration are quite unwieldy because of the extreme minuteness of H-ion concentration in weakly acid and in basic solutions. The H-ion concentration of pure water, for instance, has been shown to be 0.000,000,1 gram per litre, or in other words 0.000,000,1 normal (as a normal acid contains one gram of hydrogen per litre). A simpler method indicating this small quantity is by the logarithmic form of expression, i.e., 10^{-7} or $\log -7$. This method of expression was adopted by Sørensen, who was the first to point out the great influence of H-ion concentration (as distinct from titrable acidity) upon biological activities. Sørensen used the symbol P_H^+ (or the simpler P_H) to represent this logarithm, without the minus sign; or using mathematical language, P_H is the logarithm of the reciprocal of the H-ion concentration expressed in grams per litre. Sørensen used this symbol to signify H-ion concentration. By this method of expression, the H-ion concentration of pure water is indicated by the formula $P_H = 7.0$.

Because P_H is an invert logarithm, it decreases with increasing H-ion concentration. Therefore, acid solutions have a P_H -value smaller than 7.0, basic solutions a P_H -value greater than 7.0. It has been found, for instance, that the P_H -value for 1%

normal HCl is 1.05, for normal acetic acid 2.4, while for $\frac{1}{10}$ molecular NH_4OH it is 11.2.

In interpreting P_{H} -values it must be remembered that adding 1 to the logarithm of a number is equivalent to multiplying the number itself by 10. For example, $\log 3$ (i.e., 1000) = $10 \times \log 2$ (i.e., 100). Similarly, to use an illustration that more nearly concerns us here, $\log -6$ (i.e., 0.000,001) = $10 \times \log -7$ (i.e., 0.000,000,1). In other words, a solution with a P_{H} -value of 6 has ten times the H-ion concentration of pure water ($P_{\text{H}} = 7$); and one with a P_{H} -value of 5 has ten times the H-ion concentration of a solution with a P_{H} -value of 6, or 100 times the H-ion concentration of pure water. Thus by a simple calculation it is possible to compute the relation between the H-ion concentrations of two solutions provided the difference in P_{H} -value is an integer. In case the difference in P_{H} -value is composed of an integer and a decimal, the relation may be learned by finding the number of which the decimal is the logarithm and giving it one more place to the left of the decimal point than there are units in the integer. An example will make this clearer:

For $\frac{1}{10}$ normal HCl: $P_{\text{H}} = 1.05$

For normal acetic acid: $P_{\text{H}} = 2.4$

The difference is: 1.35

Now $0.35 = \log 2.24,$

But as the integer is 1, there must be two places to the left of the decimal point, i.e., 22.4 (approximately 22.5).

Hence tenth normal hydrochloric acid has approximately 22.5 times the H-ion concentration of normal acetic acid.

It is also possible to compute the OH-concentration for any solution of which the P_{H} -value is known, because there is a constant relation between the two expressed by the equation

$$P_{\text{H}} + P_{\text{OH}} = 14.$$

That is, P_{OH} may be determined for any solution by subtracting its P_{H} -value from 14. Thus for normal acetic acid ($P_{\text{H}} = 2.4$) the P_{OH} value is $14 - 2.4 = 11.6$.

Measurement of H-ion concentration

There are two different methods by which the H-ion concentration of a solution may be determined: the electrolytic method and the colorimetric method. The electrolytic method is generally the more accurate, and is applicable to a greater variety of solutions; but it is time-consuming and requires quite complicated apparatus. The colorimetric method is much simpler, is accurate enough for all bacteriological work and can be applied easily to any ordinary bacteriological culture medium.

The electrolytic method. The electrolytic measurement of H-ion concentration depends upon the fact that when a metal is in contact with an aqueous solution containing dissociated ions of that metal, there is an electromotive force set up between the metal and the solution, which varies according to the concentration of the ions of this metal in the solution. This electromotive force can be measured, and by means of the proper formula, the ionic concentration of this metal in the solution can be determined. Hydrogen acts like a metal in this respect when brought into electrolytic contact with an aqueous solution. This is accomplished by immersing a platinum electrode (platinum foil, coated with platinum black) in the solution and allowing a constant stream of hydrogen to bubble over the platinum. Because of the affinity of platinum for hydrogen, it takes up enough of the gas to act as a hydrogen electrode. The electromotive force established between this electrode and any aqueous solution with which it is in contact is inversely proportional to the H-ion exponent (i.e., P_H) of that solution.

The methods of measuring this electromotive force are quite complicated and are not adapted to the average bacteriological laboratory. All the average bacteriologist needs to know is that with the proper apparatus it can be measured and that the hydrogen ion exponent can be directly determined from it.

The colorimetric method. The colorimetric measurement of H-ion concentration depends upon color changes produced in certain substances (indicators) by varying acidity. Each indicator changes from one color to another between quite narrow limits of H-ion concentration, and between these two limits

every shade of the indicator corresponds to a definite P_H value. The best known indicators to the bacteriologist are litmus and phenolphthalein. Of these, litmus has little value in accurate H-ion measurements, partly because it is not a definite chemical compound, and partly because azolitmin, its chief component (even though chemically pure), varies so much in its color reactions according to the material present in the solution that little accuracy can be obtained with it. Phenolphthalein, on the other hand, gives very accurate results within the P_H -limits between which its color changes takes place; but this range ($P_H = 8.0$ to $P_H = 9.6$) is considerably to the alkaline side of neutrality. Bacteriological work, however, has special need of H-ion determinations near neutrality and in acid solutions.

In passing, it is of interest to notice that bacteriologists, in an empirical way, have realized for some time that the phenolphthalein color changes takes place in alkaline solutions. It has been found that bacteria grow better in solutions that are slightly acid to phenolphthalein, and it has lately become customary in this country to adjust media to 1 per cent normal acid to phenolphthalein. Bacteriological media have ordinarily contained Witte's peptone; and in 1 per cent solutions of this peptone, the above titre corresponds closely to the H-ion concentration of pure water ($P_H = 7.0$). This is not necessarily true of other peptones or of other media; but bacteriologists who have used peptone Witte have for some time adjusted their media practically to true neutrality.

The relation of different indicators to various points in the P_H -scale was studied by Sørensen as well as by others who have followed him. Most recently Clark and Lubs have furnished us (1917) with a beautiful series of accurate indicators whose sensitive ranges meet or even overlap and extend from $P_H = 1$ to $P_H = 10$. The relation of these indicators to H-ion concentration is shown in figure 1 and table 1. Of special interest to bacteriologists are the three indicators nearest to $P_H = 7.0$, i.e., brom cresol purple, brom thymol blue, and phenol red. Of these brom thymol blue is most useful in adjusting the reaction of media to neutrality, because it is yellow in acid solutions

and blue in basic solutions, passing through various stages of green between the points $P_H = 6.0$ and $P_H = 7.6$. At $P_H = 7.0$ it is grass-green, a shade that can be easily recognized by the eye after a little practice. As a result it is possible to obtain media that are essentially neutral, no matter what their composition may be, merely by adding sufficient acid or base so that brom thymol blue becomes grass-green when added to them.

It is plain that a rough idea can be obtained as to the H-ion concentration of any solution by simply finding which indicators give their acid color in it and which give their alkaline

TABLE 1
Color changes of Clark and Lub's indicators

INDICATOR	FULL ACID COLOR	FULL ALKALINE COLOR	SENSITIVE RANGE
Thymol blue (acid range)	Red	Yellow	1.2-2.8
Brom phenol blue	Yellow	Blue	3.0-4.6
Methyl red.	Red	Yellow	4.4-6.0
Brom cresol purple	Yellow	Purple	5.2-6.8
Brom thymol blue	Yellow	Blue	6.0-7.6
Phenol red.	Yellow	Red	6.8-8.4
Cresol red.	Yellow	Red	7.2-8.8
Thymol blue (alkaline range)	Yellow	Blue	8.0-9.6
Phenol phthalein	Colorless	Red	8.0-9.6
Cresol phthalein	Colorless	Red	8.2-9.8

color. After a little experience with these indicators it is possible to do even better than this by inspection of the shade of color produced by whichever indicator or indicators are sensitive at the P_H -value of the solution in question. Accuracy, however, can be obtained only by actual comparison with the colors produced by the indicators in solutions of known H-ion concentration. For comparison Sørensen devised a series of standard solutions varying in H-ion concentration from $P_H = 1$ to $P_H = 13$. Clark and Lubs have proposed a different series of standards that are published in a more readily available place (loc. cit.). Of special interest is their standard corresponding to $P_H = 7.0$ (neutrality). This is prepared by mixing 5 cc. of $\frac{1}{2}$ molecular K_2HPO_4 with 3 cc. of $\frac{1}{2}$ molecular NaOH and dilut-

ing to 20 cc. with distilled water. Varying the proportion of these two solutions in the mixture increases or decreases the H-ion concentration as shown by table 1 of Clark and Lubs (1917, p. 26).

The difficulty of preparing such standard solutions of known H-ion concentration in the ordinary bacteriological laboratory has led Barnett and Chapman (1918) to devise* a series of color standards that do not require accurate chemical adjustments. The standards that they propose are for phenol red, but they state that the method may be used for other indicators. Their method is to prepare two series of tubes, one series containing a

TABLE 2

Barnett and Chapman's color standards for H-ion determinations

Pair no. 1.....	5 cc. acid + 9 drops* indicator; 5 cc. base + 1 drop indicator
Pair no. 2.....	5 cc. acid + 8 drops indicator; 5 cc. base + 2 drops indicator
Pair no. 3.....	5 cc. acid + 7 drops indicator; 5 cc. base + 3 drops indicator
Pair no. 4.....	5 cc. acid + 6 drops indicator; 5 cc. base + 4 drops indicator
Pair no. 5.....	5 cc. acid + 5 drops indicator; 5 cc. base + 5 drops indicator
Pair no. 6.....	5 cc. acid + 4 drops indicator; 5 cc. base + 6 drops indicator

*In some laboratories it may prove simpler to use 10 cc. in each tube and to measure out the indicator solution in tenths of a cubic centimeter instead of in drops.

dilute acid solution, the other a dilute basic solution, adding decreasing quantities of the indicator to the acid series and increasing quantities to the alkaline series. The acid tube with the largest quantity of indicator and the alkaline tube with the smallest quantity of indicator form a pair (to be viewed together in transmitted light); the acid tube with the next largest quantity of indicator and the alkaline tube with the next smallest quantity form a second pair; and so on. This arrangement is shown in table 2.

It will be seen that the sum of the amount of indicator in the two tubes of each pair is always the same. Looking at the light through both tubes of any particular pair, the same color is obtained as when a solution of the proper H-ion concentration is viewed with an amount of indicator in it equal to the sum of

the quantity in the two tubes of the standard. The standard contains more indicator in the acid or in the alkaline tube respectively according to whether the pair of tubes is to represent the acid or the alkaline end of the range of the indicator.

Barnett and Chapman give the P_H -values corresponding to these successive pairs of tubes (using phenol red as indicator) as 6.9, 7.2, 7.5, 7.7, 7.9, and 8.1, respectively. Tested by a member of this committee they were found to correspond respectively to the P_H -values 7.1, 7.3, 7.5, 7.7, 7.8, and 7.9. Evidently the variation in the hands of different men is slight, probably less than in making up standard solutions of known H-ion concentration in the ordinary bacteriological laboratory. Such standards can be very quickly prepared even by a beginning student, and their use is therefore highly to be recommended.

The same method can be applied to brom thymol blue, thus covering a range more useful to some bacteriologists. The six pairs of brom thymol blue standards, prepared as given in table 2, correspond (as tested by a member of this committee) to the P_H -values 6.2, 6.4, 6.7, 6.9, 7.1, and 7.3, respectively. These two indicators together thus furnish standards covering the range from $P_H = 6.2$ to $P_H = 8.0$ with duplication at 7.1 and 7.3.

Why the titration method is illogical

The conventional method of adjusting the reaction of media or of determining the amount of acid produced by cultures depends upon titration to phenolphthalein. A computation is made of the per cent of normal alkali necessary to neutralize the solution, and the acidity is stated as equivalent to this per cent of normal acid. The assumption upon which this procedure is based is that the reaction of the solution is measured by the amount of base necessary to neutralize. This assumption is incorrect. Ten cc. of N/10 HCl and 10 cc. of N/10 acetic acid each require 10 cc. of N/10 NaOH to be neutralized, but the HCl is much more acid than the acetic acid.

In bacteriological media, moreover, there are nearly always present certain materials which have a buffer effect. Although

but weakly acid, they combine to a marked extent with the base used in titration and thus prevent the neutralization of the solution as rapidly as would be the case if no buffer were present. Peptone, for example, has a very strong buffer effect upon solutions in which it is present. As a result, in peptone solutions which are actually neutral, the titrable acidity may be quite high, because the NaOH used in titration combines with the peptone without greatly lowering the reaction of the solution. Hence titration is a very illogical method of determining the reaction of solutions containing peptone.

An example may make this clear. Witte's peptone is about neutral and its solutions are grass-green to brom thymol blue; but in a 1 per cent solution it titrates about 1 per cent normal acid to phenolphthalein. Difco peptone is more acid, giving a greenish yellow with brom thymol blue; but as it does not have such a marked buffer effect, it titrates lower than peptone Witte. As a result, now that Difco peptone is often used in America in the place of Witte peptone, it is not infrequent to find someone adding acid to it to bring it to 1 per cent normal to phenolphthalein, whereas alkali should be added to it to make it neutral to brom thymol blue. It should further be noticed that any peptone has practically the same H-ion concentration no matter what the concentration of the solution may be; but the titrable acidity is lower the more it is diluted. Hence the titration method leads to the error of adding acid to a 0.1 per cent solution of peptone to make its "reaction" the same as that of a 1 per cent solution.

A further fault of this titration method is that there is no sharp phenolphthalein neutral point. This indicator begins turning red at about $P_{\text{H}} = 8.0$ but does not reach its full alkaline color until about $P_{\text{H}} = 10.0$. Some bacteriologists titrate until its full color has appeared, others stop as soon as the first appearance of pink is evident, while still others try to use a point half way between these two limits. The result is considerable variation in the hands of different men.

All of these objections make the titration method entirely illogical for adjusting the reaction of media or for determining

the amount of acid produced by an organism. The colorimetric method of determining H-ion concentration is more logical, is of more significance, and is really more simple after the principles of the technic are once learned.

IV. THE REDUCTION OF NITRATES

The ordinary method of testing for nitrate reduction—as recommended in the last report of this committee (J. Bact., 3, 124)—is to grow the organism in a liquid nitrate medium and to test for nitrite after a definite length of incubation. The presence of gas is to be recorded if observed. On previous charts adopted by the Society the determination of ammonia was also called for, provision for making this determination having been made in the reports of the committees on water analysis of the American Public Health Association. It was distinctly stated in these reports, however, that ammonia may come from the peptone instead of from the nitrate, so that presence of ammonia in absence of nitrite does not necessarily indicate nitrate-reduction. This latter source of error, as has been pointed out by one member of this committee (Kligler, 1913), is greater than has been generally recognized by bacteriological writers. Hence no provision for the ammonia test is made on the latest chart recommended by the committee.

Omitting the ammonia test, however, opens up the possibility of error in another direction, for some organisms convert the nitrite into ammonia as rapidly as it is formed, so that its presence can scarcely be detected. This matter will shortly be further discussed by H. J. Conn and R. S. Breed in a paper (now in press) to appear shortly in this Journal. The nitrate-reduction test proves, indeed, to be a far more complicated matter than originally supposed. Absence of nitrite may mean any of the following things: (1) actual inability to reduce nitrate, (2) a medium so poorly adapted to the organism in question that growth is poor or lacking, (3) conversion of nitrite into ammonia as fast as produced, (4) assimilation of the nitrite-nitrogen (either as nitrite or as ammonia) as fast as

produced. Obviously a qualitative test for nitrite fails to distinguish between these four very different possibilities. A nitrite test accompanied by a quantitative ammonia test would undoubtedly prove an aid in detecting those organisms that reduce nitrate without an accumulation of nitrite; but as yet the committee has not worked out a quantitative test for ammonia simple enough for the bacteriological laboratory. Sometimes it is possible to obtain data of more significance by means of a qualitative test for ammonia, provided an ammonia-free medium is used containing no nitrogen other than the nitrate. In such a medium the only source of ammonia is the nitrate and its presence indicates nitrate-reduction even though nitrite be absent. As it is difficult, however, to obtain such a medium in which the majority of bacteria will grow, this method is realized to be of value only in special cases.

As a result the committee is unable at present to propose a standard method for the determination of nitrate-reduction. The provisional procedure recommended is as follows:

Inoculate first into nitrate broth and onto slants of nitrate agar. For this purpose use the standard formula for beef-extract broth (page 115 of 1917 report) with the addition of 0.1 per cent KNO_3 , instead of the formula for nitrate broth given on p. 124 of the 1917 report. The richer medium is better adapted to pathogenic and to many other bacteria, and its greater viscosity aids in the detection of gas bubbles in case free nitrogen is produced. Test both the broth and the agar culture for nitrite by means of the reagents given on p. 124 of the 1917 report. (These reagents give the nitrite reaction as readily if poured on the surface of an agar slant as if added to a liquid culture). Presence of nitrite or of gas (indicated by foam in the broth or by cracks in the agar) shows the nitrate to have been reduced. A negative result does not prove that the organism is unable to reduce nitrates; in such a case further study is necessary, as follows:

In case the fault seems to lie in poor growth, search for a nitrate medium in which the organism in question does make good growth by means of the following modifications: increas-

ing or decreasing the amount of peptone; altering the reaction; adding some readily available carbohydrate. Presence of nitrite or gas in any nitrate medium whatever should be recorded as nitrate-reduction.

If the organism grows well and yet produces no nitrite or gas, the determination must be recorded as doubtful unless the organism can grow well in some synthetic medium containing no nitrogen except nitrate. It is recommended that such an organism be tested in a medium containing small quantities of phosphate, calcium, chlorine, etc., with KNO_3 as a source of nitrogen and sucrose as a source of energy and of carbon.³ Such a medium generally allows good growth with an organism capable of utilizing nitrate and sucrose. Unfortunately neither glucose nor lactose can be used in this medium as a source of carbon and energy, for the ordinary "c.p." preparations of these sugars contain much ammonia. If the organism in question grows (even but slightly) on a synthetic medium of this sort, it should be tested for nitrite by the usual method and for ammonia by means of Nessler's reagent (comparing with an uninoculated tube as a check). The presence of nitrite, of ammonia (i.e., a more pronounced ammonia reaction than in check tube), or of gas indicates nitrate-reduction.

The production of gas (free N) from nitrate is not a very common one; but a considerable number of soil organisms have this power, and one should be on the lookout for it in studying soil bacteria. The agar slant test is ordinarily a sufficiently delicate test; but, if liquid media are used, more reliable results may be obtained by the use of fermentation tubes.

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³ An illustration of such a medium which has proved satisfactory for some bacteria is: K_2HPO_4 , 0.5 gram, CaCl_2 , 0.5 gram, KNO_3 , 1 gram, sucrose 10 grams, agar 12 grams, water 1000 cc.

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BACTERIAL VARIATIONS INDUCED BY CHANGES IN THE COMPOSITION OF CULTURE MEDIA

ANDREW IGNATIUS DAWSON

From the Department of Bacteriology, Columbia University, New York City

Received for publication, May 11, 1918

INTRODUCTION

It is to be regretted that the tendency of investigation in bacteriology has been largely toward a study of effects, with little or no attempt to explain their fundamental causes. This is probably because the latter problems involve greater technical difficulties and offer less hope of immediate solution. Until a definite scientific foundation has been built up, however, on which to correlate the numberless facts already known, these will always remain isolated and to some extent unexplainable.

Bacteria, like all living cells, produce changes in internal composition as well as in external environment by means of chemical reactions. Living protoplasm, in order to maintain its viability, is constantly abstracting certain essentials from its environment, and replacing them by other substances, while it is, itself, in a constant state of alteration.

The changes wrought in the surrounding material by the presence of bacteria can only be definitely, that is, chemically, followed by cultivating them upon media of known and exactly reproducible composition, the so-called synthetic media. Suitable media of this type are not easily developed, but work is in progress along this line which cannot fail to bring to light many significant and fundamental relationships.

Changes within the bacterial bodies are even harder to follow. The present means which analytical chemists have at their disposal in the investigation of the most complicated of all known forms of matter, are palpably imperfect. Bacterial changes,

however, may be at times accompanied by (1) alterations in the empirical chemical composition of the whole bacterial bodies, and (2) alterations in the biological behavior of the organisms. Alterations in both these quanta are demonstrable, and can, at least in part, be correlated with their etiological factors.

In this paper we have attempted to add something to the data regarding the extent of alteration in the empirical composition of bacteria, to show how such alterations may be brought about, and to draw certain conclusions regarding their probable causes. We hope at least that the data here presented will be useful to future investigators in this field.

To produce a determinable change in either the properties or composition of bacteria, it is necessary to change their environment. This is easily done by first altering the nature of the medium upon which they are grown. By regulating the transplants with sufficient frequency to make sure that the changes observed are not due to bacterial "old age," and by continuing cultivation on the altered medium long enough to produce a reasonably complete adaptation of the organism to its new environment, the experiments can be adequately controlled, and an observable change of properties and composition, if such is possible, can be obtained.

The determination of the chemical composition of bacterial bodies is not an easy task. Ordinary bacterial growths are actually composed of very small quantities of material, and the production of sufficient bacterial substance for a chemical analysis is difficult.

PRELIMINARY PROCEDURE

In order to determine the biological change in bacteria in any direction, it is necessary (1) to preserve the same organism throughout all determinations, and (2) to determine the total activity of the organism in statu quo as far as possible, and (3) lastly, to subject the organism to constant varied conditions.

In accordance with these requirements, a strain of *B. coli*, which showed ready and abundant growth, was selected and preserved in all the following operations. It had been pre-

viously cultured on meat-extract agar for an unknown number of generations, of which at least 250 are known. In regard to this culture two questions present themselves: old age, and adaptation. If an organism is in a state of decrepitude, a transfer to another medium may mean only rejuvenation, which should assert itself in the next generation on any different medium on which the organism could have wide scope for its metabolic processes. A test of this possibility was made as follows: A twenty-four hour culture of the organism was emulsified in sterile salt solution of which a measured volume was plated on two different media: (1) meat-extract agar, and meat-infusion-glucose agar. After twenty-four hours' incubation the former showed 75 colonies, the latter showed 63. There seemed to be no distinguishing characteristic differentiating the organism from the colonies of the two plates. From this experiment the question of old age in this strain may be considered as eliminated.

The laws of adaptation, on the other hand, govern the internal state of the organism in its present conditions either fully or only partially. If the organism is fully habituated to its environment, a substantial change of environment effects a growth which is sparse; or, really or apparently abundant; or, no growth at all appears. If the abundant growth is apparent, and not real, it will be transitory; if real, it will persist.

Whether bacteria obey the laws of adaptation fully, partially, or not at all, may or may not be proven by external phenomena; but since the question should receive some answer from an examination of internal protoplasmic conditions, *a priori* an organism confined to one environment for many generations ought to throw light upon this point. The exact number of generations that will influence bacteria in this direction has not been determined. Arbitrarily, therefore, the 200th generation was chosen to initiate the solution of the question. In all the following experiments the organism had been confined to one medium for at least 200 generations before it was finally grown on this medium for examination.

In order to determine the biological activity of the organism in its present environment, it was cultured on a medium to which

it had been accustomed, i.e., agar, 2.5 per cent; peptone, 1 per cent; NaCl, 0.5 per cent; meat-extract, 1 per cent; incubated in test tubes 8 by 1 inch at 37°C. Under these conditions growth after twenty-four hours was fairly abundant, and even seemed to increase after that period.

To prove approximately an increase or decrease in growth after twenty-four hours' incubation, 100 tubes of different ages were selected, the planted area of which seemed to be of the same dimension. The growth was scraped from the medium at twenty-four hour intervals, transferred to weighing bottles

TABLE I

DAYS' GROWTH	APPROXIMATE WEIGHT OF BACTERIAL GROWTH IN 100 TUBES			APPROXIMATE WEIGHT OF BACTERIA AFTER DESICCATION			APPROX. AVERAGE PERCENTAGE LOSS BY DESICCATION
	I	II	III	I	II	III	
1	2.0	1.7	2.3	0.05	0.05	0.07	97
2	2.1	1.9	2.4	0.06	0.06	0.07	97
3	2.1	2.0	2.5	0.10	0.10	0.13	95
4	2.7	2.4	2.7	0.25	0.24	0.28	90
5	3.2	2.7	3.3	0.54	0.47	0.56	83
6	3.2	3.0	3.6	0.54	0.51	0.60	83
7	5.3	3.7	4.5	0.90	0.63	0.77	83
8	5.9	3.9	5.8	1.30	0.86	1.30	78
9	5.9	4.5	6.3	1.30	1.00	1.40	78
10	6.2	4.6	6.5	1.60	1.20	1.80	74
11	6.5	4.6	6.8	1.70	1.20	1.80	74
12	6.3	4.6	6.8	1.70	1.20	1.80	73
13	6.3	4.4	6.8	1.70	1.20	1.80	73
14	6.0	4.2	6.7	1.70	1.20	1.80	72

and weighed. After weighing, the material was carefully preserved at 0 to 5°C. for further examination. Growth of nine to eleven days proved to be the maximum.

In as much as the presence of moisture affects the weight of the collected bacteria, it is necessary to determine what relation moisture bears to the total weight. This determination will decide whether, and to what degree, the growth has been really or only apparently increased. Determinations of the water content were, therefore, made by drying the collected organism in weighing bottles at 97°C. over calcium chloride to constant

weight, which required seven days, and cooling the dried material in vacuo over the same dryer.

Experiments in this direction indicate that what holds true for bacterial derived substances,¹ also holds true for the whole bacterium, e.g., that bacteria show a great avidity for water. The water content of bacteria, therefore, seems to be subject to variations dependent on the period of incubation. The variation does not seem to depend on mere mechanical adhesion, because: First, of the difficulty attendant on desiccation; Second, of the power of absorbing moisture which dried bacteria or bacterial substance display to such an extent that three days' exposure to an ordinarily dry atmosphere increases the weight of the desiccated material 0.05 per cent.

It may not be contended that the entire loss of weight by desiccation is due to water alone, since bacteria may contain other volatile substances. The loss of volatile weight keeping pace with the age of the culture may be interpreted to mean that the organism in an environment of abundant food may need more than the ordinary supply of water, so that, the food decreasing, there is a decrease in the amount of water absorbed.

CULTURAL CHARACTERISTICS

Since the characteristics of the organism as grown on its accustomed medium are to be compared with those in a changed environment, a summary of the normal characteristics is necessary. In morphology, staining, and cultural traits, this organism showed only what is ordinary to *B. coli*. On Hiss' carbohydrate-serum-water medium, it produced acid and gas in glucose, levulose, galactose, maltose, sucrose, dextrin, and mannit.

BIOCHEMICAL CONSTITUENTS

Preliminary. The biochemical constituents are here understood as water and volatile matter; ash and ash constituents; sulphur; phosphorus; calcium; total nitrogen; amino-nitro-

¹ Leach, Journ. Biol. Chem., 1906, 1, 463.

gen; fats and waxes; coagulable protein; soluble protein; carbohydrates; cellulose-like substances; residue.

In making determinations of this nature on bacteria, some of the well established methods are obviously inapplicable, and less well recognized ones had to be adopted, e.g., for extraction of fat. This necessitated, in such cases, before all else a careful investigation of the method.

DISCUSSION OF METHODS

Water and volatile matter were determined only by desiccation.

Ash. The material was incinerated in a silica crucible and kept at a dull red glow to constant weight; cooled in vacuo and weighed. *Constituents* of ash were subjected to ordinary qualitative and quantitative methods.

Sulphur. Total sulphur was estimated on both wet and dried bacteria. The material was heated with sodium carbonate and an excess of potassium nitrate, extracted with water, neutralized, and after precipitation weighed as barium sulphate.

Loosely combined sulphur was estimated as sulphide. The material was boiled four hours in 10 per cent KOH, neutralized with acetic acid, and precipitated as lead sulphide, care being taken during the process to prevent the formation of lead sulphate or carbonate.

Phosphorus. The material was first decomposed by boiling nitric acid, precipitated by ammonium-phospho-molybdate, redissolved in ammonium hydroxide, reprecipitated and estimated as magnesium pyro-phosphate.

Calcium. The material was decomposed by boiling sulphuric acid, precipitated by absolute alcohol, washed with 40 per cent alcohol at 34°C., and weighed as sulphate.

Total nitrogen. This was estimated by the macro-Kjeldahl method.

Amino-nitrogen was determined by the Van Slyke method.

Coagulable protein

Preliminary. Since a quantitative estimation of the protein content of the bacterial cell would assist in determining biological variations, the ideal method of protein extraction would involve the disintegration of the cell material without injury to the proteins. But the cellulose-like structure of the cell presents interference. To dissipate this, two alternatives are offered; to employ a cellulose enzyme, or to carry on extraction as far as possible by means of chemical reagents. Enzymes, however, are objectionable. Chemical reagents therefore must be resorted to, success depending on proper selection.

In exploring the effect of acids, alkalis, and neutral salts and solvents on proteins, it appeared that distilled water and sodium chloride possess greatest efficiency especially at reduced pressure. Of the two solvents, distilled water was discarded because it extracts fats together with proteins, and sodium chloride was selected as being a more powerful protein solvent.

The method of determining quantitatively the protein content of bacteria was therefore the following: Weighed amounts of wet bacteria were subjected to extraction by a definite amount of 6 per cent NaCl for twenty-four hours at 50°C. under 250 mm. pressure. After sedimentation, decantation of the supernatant liquid followed. The sediment was washed, and after the wash waters had been added to the original liquid, the whole was reduced to its original volume by evaporation. Part of this solution was weighed to ascertain the approximate protein content.

This solution heated to 85 to 90°C, with acetic acid to a concentration of 0.01 per cent, produces a coagulum, which when washed and dried on a weighed filter, is estimated as *coagulable protein*. The filtrate from the previous operation, after being diluted to 0.1 per cent NaCl was divided into two parts; one part was precipitated by HCl 0.1 per cent, and the other part by 0.1 per cent KOH. The precipitates were washed, dried, and weighed as *acid-precipitable* and *alkali-precipitable protein*.

The filtrates from the acid- and alkali-precipitations still gave evidence of containing protein not easily precipitated by mineral acids, alkalis, and neutral reagents. After neutralizing, the

protein content of these filtrates was estimated by Esbach's method, and listed as *soluble proteins*.

The insoluble residue containing a protein which resisted extraction, was subjected to tryptic digestion; the difference in weight before and after digestion was recorded as *insoluble protein*.

The substance remaining after the last operation, having given no protein reaction, nor showing the presence of nitrogen, was reserved for other examinations as *residue*.

Fats

Preliminary. Determinations of fat could not be made with accuracy until the known methods were examined. Experiments showed fat to be at least partly intracellular, and consequently total disintegration of the cell was necessary. The liberation of fat without its deterioration after cell disintegration was effected by means of alkaline hypochlorite, commercially known as 'antiformin.'

Method. Weighed quantities of wet bacteria were treated with 20 per cent antiformin for twenty-four hours at 30°C., then warmed to 60°C. for one hour. After cooling to 15°C., and neutralizing with N/10 sulphuric acid, the solution was evaporated to dryness at 80°C., ground, and extracted with ethyl- or petrol-ether.

This residue was slowly boiled in 10 per cent NaOH, and after boiling, was rendered acid with dilute sulphuric acid. It was then diluted to three times the original volume with distilled water and cooled to 7° to 10°C., after which it was filtered. The precipitate was washed, dried, and extracted for five hours with petrol-ether.

Carbohydrates

Preliminary. When the bacterial residue, after protein extraction, is treated with concentrated sulphuric acid at ordinary temperature, it becomes turbid, and dark brown, finally turning

to clear deep red. If this reaction is hastened by gentle heat to 40 to 45°C. for one hour, diluted with 5 per cent KOH, and neutralized, the solution gives a reaction for reducing substances.

If the same process be carried on at a lower temperature, the activity of the acid is decreased, as is evidenced by the color, but the *amount* of reducing substance seems to increase. Also, whether normal bacteria or bacterial residue be employed in this process, it happens that when the reaction has proceeded to the production of a deep red coloration without turbidity, if cold distilled water be slowly added, a heavy gelatinous precipitate settles out of the solution. After neutralizing, this solution may be filtered and the precipitate recovered. The amount of reducing substances under these conditions is reported as *Carbohydrate* in terms of glucose.

The precipitate here obtained, after being washed by decantation, was found to yield a brown color with iodine, and not to be colored blue by iodine-zinc chloride. It is soluble in ammoniacal cupric oxide (Schweitzer's reagent), reprecipitated by acids, and is soluble in zinc chloride, from which it can be recovered on dilution with water. On hydrolysis with sulphuric acid reducing substances are formed.

These characteristics are essentially those of cellulose, and we regard the material so obtained as similar to, if not identical to that substance. The presence of cellulose in bacterial bodies has been a much discussed question, as throwing possible light on the vegetable nature of bacteria. The findings of Vaughan² seem to indicate that cellulose is absent in bacteria, but we are inclined to consider our results as confirming those of Dreyfuss, Hammerschlag, and others.³

The precipitate here obtained, and quantitatively estimated by collecting it on a weighed filter and weighing, was, therefore reported as *cellulose-like substance*.

² Prot. Split Prod., pp. 66-67.

³ Zeitsch. fur physiol. Chem., 1887, 9, 181.

DETERMINATIONS

After experimental examination, the above methods were adopted uniformly and without modification.

The material for these determinations was a nine days' growth of *B. coli*, collected at the stage of maximum development.

The media employed for obtaining growth, and representing change of environment, are described in the adjoined table 2.

Bacteria collected from these several media constitute the material for analyses by the methods already indicated, the number of determinations in each analysis varying from 7 for

TABLE II

Media employed in obtaining nine days' growth of B. coli for analysis

	SOLID MEDIA: VEHICLE, AGAR 2 PER CENT	TITRATION
I	Peptone, 1 per cent; meat extract, 1 per cent	Neutral
II	Peptone, 0.5 per cent, edestin 0.5 per cent.....	Alkaline
III	Peptone, 0.25 per cent, flour proteins, 1 per cent.....	Alkaline
IV	Peptone, 0.25 per cent, meat extract, 1 per cent, glucose, 1 per cent	Neutral
V	Peptone, 0.25 per cent, meat extract, 1 per cent, glucose, 1 per cent glycerol, 1 per cent	Neutral
VI	Peptone, 0.25 per cent, butter soap, 1 per cent..	
VII	Peptone, 0.25 per cent, butter soap 1 per cent.....	
VIII	Peptone, none, potato juice, from whole unskinned potato, freed from starch, 500 grams potato to litre of medium...	

ash to 20 for nitrogen. For the sake of brevity 3 of the most variant results are indicated in table 3.

Since table 3 does not include the results of all determinations, but only the most variant, for the purpose of comparison the average result for each series of determinations is therefore appended.

Table 4 presents variations in almost all bacterial constituents. Though strict interpretation of these results must necessarily await the light of further research, attention must be directed to the fact that from the simplest to the most complex constituents, variation is evident. The water content varies, which may not

TABLE III

Data pertaining to the analysis of B. coli grown on media I to VIII. Determinations and calculations are based on wet bacteria

	MEDIUM							
	I	II	III	IV	V	VI	VII	VIII
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Water and volatile matter.....	74.45	72.52	60.28	75.10	75.00	60.70	60.36	79.62
	74.83	72.50	60.22	75.08	74.83	60.66	60.31	79.56
	74.86	72.43	60.20	75.00	74.96	60.60	60.26	79.50
Ash.....	4.80	2.73	2.53	4.51	4.50	7.84	7.69	2.14
	4.80	2.70	2.50	4.51	4.50	7.83	7.69	2.11
	4.80	2.69	2.44	4.49	4.50	7.83	7.67	2.03
Sulphur (total).....	0.06	0.00	0.00	0.11	0.10	0.00	0.00	0.16
	0.05	0.00	0.00	0.09	0.10	0.00	0.00	0.14
	0.05	0.00	0.00	0.08	0.09	0.00	0.00	0.11
Sulphur (loosely combined).....	0.00	0.00	0.00	0.02	0.01	0.00	0.00	0.03
	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.03
	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.02
Phosphorus (as P_2O_5)..	4.25	3.50	2.38	2.90	3.31	1.71	1.83	0.92
	4.25	3.48	2.38	2.88	3.29	1.69	1.83	0.92
	4.22	3.47	2.37	2.87	3.28	1.69	1.83	0.92
Calcium (as CaO).....	2.71	0.05	1.07	2.62	2.61	2.34	2.35	0.19
	2.67	0.05	1.06	2.62	2.60	2.33	2.33	0.18
	2.63	0.04	1.06	2.61	2.59	2.34	2.33	0.18
Nitrogen (total).....	2.844	3.009	6.227	2.419	2.120	4.330	5.012	5.030
	2.842	3.012	6.222	2.410	2.113	4.330	5.009	5.026
	2.841	3.022	6.219	2.400	2.109	4.328	5.003	5.023
Nitrogen (amino-).....	0.780	0.930	2.976	0.730	0.693	1.721	1.651	3.016
	0.777	0.887	2.969	0.721	0.690	1.689	1.650	3.011
	0.774	0.874	2.968	0.718	0.687	1.659	1.650	3.007
Protein, coagulable.....	2.99	1.00	4.70	2.35	2.50	4.10	5.35	5.57
	3.02	0.98	4.65	2.33	2.45	4.07	5.35	5.56
	2.98	0.98	4.65	2.31	2.44	4.03	5.31	5.53
Protein (acid-precipitable).....	7.48	6.65	9.60	7.37	4.67	6.53	6.90	2.14
	7.45	6.62	9.56	7.30	4.58	6.49	7.01	2.09
	7.40	6.58	9.53	7.28	4.55	6.41	6.89	2.03

TABLE III—*Continued*

	MEDIUM							
	I	II	III	IV	V	VI	VII	VIII
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Protein (alkali-precipitable).....	5.64	7.50	2.89	6.20	5.41	6.72	6.84	4.29
	5.61	7.47	2.83	6.01	5.32	6.63	6.79	4.24
	5.58	7.39	2.76	6.00	5.28	6.57	6.79	4.19
Protein (soluble).....	0.06	2.20	1.47	0.09	0.14	0.09	1.22	1.14
	0.05	2.27	1.30	0.09	0.11	0.09	1.21	1.08
	0.04	3.01	1.22	0.05	0.08	0.08	1.21	1.07
Protein (insoluble) . . .	0.05	0.05	0.10	0.07	1.01	0.08	0.13	0.04
	0.05	0.03	0.08	0.05	0.98	0.06	0.09	0.02
	0.05	0.03	0.08	0.03	0.98	0.06	0.09	0.02
Residue (insoluble).....	1.03	2.40	2.05	1.43	1.76	1.00	1.52	0.81
	0.99	2.39	2.01	1.39	1.69	0.97	1.48	0.79
	0.99	2.32	2.00	1.39	1.63	0.95	1.43	0.75
Fats.	4.06	4.40	4.91	5.80	8.01	*	*	5.10
	4.01	4.34	4.82	5.78	8.00			5.06
	3.98	4.29	4.78	5.75	8.00			5.05
Carbohydrate.....	4.03	3.13	2.22	1.41	2.73	3.06	2.95	1.04
	4.00	3.10	2.19	1.39	2.68	3.00	2.90	1.00
	3.97	3.09	2.17	1.35	2.66	2.97	2.86	0.97
Cellulose-like substance.	1.03	2.45	2.06	1.46	1.79	1.10	1.55	0.90
	0.99	2.41	2.01	1.40	1.76	1.08	1.51	0.83
	0.98	2.39	1.97	1.37	1.70	1.07	1.49	0.76

*Not determined, because of extraneous fat attached to bacteria.

be as significant as other variations. There is an increase in the amount of ash and amino-nitrogen in the case of fat-media; protein is high where protein is present in the medium and highest where the medium-protein is in soluble form. Differences in quantity of coagulable protein show that the bacterial protein is, in many instances, itself different. This is supported by the fact that the ratio of nitrogen to any one of the protein fractions, or to all of them combined, is not constant. Also, it can be seen that the presence of certain sub-

TABLE 4

Summary of results obtained by use of different media in the cultivation of B. coli

	MEDIUM							
	I	II	-III	IV	V	VI	VII	VIII
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Water and volatile matter.	74.84	72.5	60.25	75.01	74.9	60.69	60.32	79.55
Ash.....	4.8	2.7	2.5	4.5	4.5	7.83	7.69	2.1
Sulphur (total).....	0.06	0.0	0.0	0.1	0.09	0.0	0.0	0.14
Sulphur (loose).....	0.0	0.0	0.0	0.02	0.01	0.0	0.0	0.03
Phosphorus P ₂ O ₅	4.24	3.48	2.38	2.89	3.30	1.69	1.83	0.92
Calcium CaO.	2.66	0.05	1.06	2.62	2.60	2.34	2.34	0.19
Nitrogen—Total.....	2.843	3.02	6.22	2.405	2.115	4.327	5.005	5.027
Nitrogen—Amino . . .	0.771	0.891	2.970	0.724	0.691	1.696	1.650	3.012
Protein, coagulation.....	2.99	0.97	4.66	2.34	2.47	4.05	5.32	5.54
Protein, acid-precipitable.	7.42	6.61	9.57	7.32	4.61	6.47	6.93	2.08
Protein, alkali-precipitable	5.60	7.44	2.80	6.05	5.33	6.63	6.80	4.22
Protein, soluble	0.05	2.25	1.31	0.07	0.10	0.08	1.21	1.10
Protein, insoluble. . . .	0.05	0.04	0.09	0.06	0.99	0.07	0.10	0.03
Residue, insoluble.....	1.00	2.37	2.00	1.40	1.70	0.98	1.49	0.76
Fats.	3.99	4.32	4.82	5.77	8.00	*	*	5.07
Carbohydrate	4.00	3.10	2.19	1.38	2.69	3.01	2.88	1.00
Cellulose-like substance..	1.00	2.42	2.01	1.41	1.75	1.09	1.52	0.81

* Not determined because of extraneous fat attached to bacteria.

stances in the environment, particularly fats, alters the chemical constitution of the organism.

BIOLOGICAL VARIATIONS

As the chemical constituents of the bacterial cell seem to be affected by a change in medium, so does it appear that the biological characteristics alter. Having seen that bacteria vary as stated above in regard to chemical composition when grown on different media, it seemed desirable to determine if such variation was accompanied (as might be reasonably expected) by variations in the biological activities of the organism.

From those biological characteristics that can be definitely measured, we have selected (1) the ability to produce enzymes capable of splitting different carbohydrates with the formation of gas and acid; and (2) the ability to agglutinate with immune rabbit serum prepared with the organism as antigen.

CARBOHYDRATES

The organism (*B. coli*) was grown on the 8 different media used above, and after about 200 generations was transplanted directly in every case to tubes of litmus-carbohydrate-serum-water, in which the production of acid and gas was noted. The time of the appearance of the phenomena in each case is recorded in table 5.

TABLE 5

Results of *B. coli* grown on 8 different media and transferred to litmus-carbohydrate-serum-water. "A" indicates hours required to produce marked acidity to litmus. "G" indicates hours required for first appearance of gas

CARBOHYDRATES	MEDIUM															
	I		II		III		IV		V		VI		VII		VIII	
	A	G	A	G	A	G	A	G	A	G	A	G	A	G	A	G
Lactose.. . . .	24	24	24	24	18	18	10	10	10	10	10	10	10	10	48	48
Mannit	24	24	24	24	24	24	18	18	18	18	10	10	10	10	48	48
Sucrose.....	24	24	36	72	36	0	24	24	24	24	0	0	0	0	48	48
Maltose.....	24	24	18	18	18	18	18	18	18	18	18	18	10	10	36	36
Levulose.....	24	24	24	24	24	24	24	24	12	12	5	5	5	5	48	48
Glucose.....	24	24	24	24	24	24	24	24	24	24	5	5	5	5	36	36
Galactose.....	24	24	18	18	18	18	12	12	12	12	5	5	5	5	40	40
Dextrin.....	48	48	36	36	30	30	24	24	30	30	10	10	10	10	56	56

Since the organisms were originally the same, variations observed in the carbohydrate media are referred to changes of the bacteria during growth in the 8 media selected. From this table it is seen that great variations in the enzyme production of bacteria are produced by appropriate changes in the environment. This is evidenced by the widely differing results obtained by the organism after becoming accustomed to different metabolic conditions. The organism from medium I behaves precisely as a *B. coli-communior*, while from media VI and VII it possesses almost the type characteristics of a *B. coli-communis*. Although bacteriologists have been accustomed to regard them as distinct species, we have been able, by the aid of fatty acids in the culture medium, to obtain what practically amounts to a transposition from one to the other.

AGGLUTINABILITY

In order to test variability, if any, in the agglutinability of the organism after removal from the 8 specified environments, sera were prepared from rabbits injected with the organisms grown on media I, III, V, and VII. The sera were then used in producing agglutination of the organism grown on all the different media. The results are given in table 6.

TABLE 6

Agglutinating organism (B. coli) grown on

AGGLUTINATING ORGANISM AS ANTIGEN GROWN ON MEDIUM	MEDIUM							
	I	II	III	IV	V	VI	VII	VIII
I	400	400	400	400	400	400	400	400
III	500	500	800	600	700	400	400	700
V	450	450	630	700	850	700	750	500
VII	600	600	800	600	800	900	900	700

The fact that almost the same agglutination was observed in every case shows by the most conclusive test we know that serologically the organism retained the properties of the colon bacillus. The organism presents the most active antigenic properties when grown on a medium containing fat, the least when grown without protein. The maximum agglutination generally takes place when the antigenic and agglutinated organism are grown on the same medium.

This establishes a very definite proof of the constitutional change in the organism. The differences observed in agglutinability are easily as great as those frequently utilized to demonstrate the existence of different "strains" of the same basic organism.

Since antigenic differences appear to be produced so readily, it seems to us reasonable to suppose that they can be found in all bacteria, and can be referred to the immediate past environment of the organism: further real information they do not disclose. These findings warn us, on the other hand, that bac-

teria are capable of change: consequently, if the slightly variable strains, frequently kept for investigation in laboratories, remain on the same medium for a long period, they can be expected to lose their differential characteristics, exhibiting only those that are common to all the strains.

Connected with these fundamental changes we have sought evidence of morphological alteration. The organism grown on medium I showed a bacillus about 2 micra long, in thickness one-quarter its length; on medium III, generally 3 to 5 micra long, almost 1 micron thick; on medium VII, 3 to 5 micra long, more than 1 micron thick. The true significance of these morphological changes must be determined by the variations already noted.

GENERAL CONCLUSIONS

We have shown that by changing the character of the media upon which a given variety of bacteria is grown, the chemical constitution of the bacteria can be made to vary, and with it, certain biological characteristics such as agglutinability with immune serum and the formation of sugar-splitting enzymes. In some instances the variations practically amount to the "production" of a new strain. Accompanying morphological changes appear to be relatively unimportant.

THE VALUE OF A COOKED MEAT MEDIUM FOR ROUTINE AND SPECIAL BACTERIOLOGY¹

W. L. HOLMAN

*From the Pathological Laboratories, University of Pittsburgh, Pittsburgh,
Pennsylvania*

Received for publication, July 18, 1918

The use of animal tissues added to media for the cultivation of bacteria is by no means new. The value of fresh sterile tissue added to various media has been clearly demonstrated for the growth of anaerobic microorganisms as in the method of Theobald Smith. The object of this paper is to bring to the attention of bacteriologists the cooked meat medium, now so widely used in the study of the anaerobic bacteria of war wounds, and to show its usefulness in routine bacteriological study as well as in more special lines of research.

Theobald Smith in 1890 first made use of unheated animal tissue for anaerobic growth. In 1905 Tarozi, as Theobald Smith says, "rediscovered the method." He used 1 cc. pieces of tissue added to tubes of ordinary broth and found that he could heat the tubes to boiling for under five minutes and still get anaerobic growth. Tarozi further showed that bouillon, made from infusion, heated, tubed without filtering and sterilized at 104° to 105°C. for fifteen minutes, gave growth of obligatory anaerobes, while the filtered bouillon did not. The albuminous clot was decolorized during the sterilization and following the growth of the anaerobes became more or less red. In ten to fifteen days this unfiltered bouillon loses its ability to grow the anaerobes. If heated to 110° the medium is usually unfavorable. This author also found that by adding sterile tissues to ordinary bouillon and removing it at the end of some hours, the conditions for anaerobic growth were present in the bouillon.

¹ Read before the American Association of Pathologists and Bacteriologists, Philadelphia, 1918.

In 1899 von Hibler employed an emulsion of brain in water using open tubes and prolonged steam sterilization. He noted differences in the color changes resulting from the growth of various anaerobes and used the medium to divide these bacteria into two groups. One of these groups caused blackening of the brain emulsion and produced alkali while the other left the color unchanged and formed acid.

In his extensive study of the pathogenic anaerobes in 1908 von Hibler gave fuller details of the use of this brain medium. It is made of brain finely minced, mixed with from one-third to one-fourth its volume of water or normal salt solution, tubed in tall tubes and sterilized in flowing steam for one and a half hours. He had used this medium since 1894 and emphasized the advisability of boiling for half an hour just before seeding. He explained the anaerobic conditions as being due to slow reabsorption of oxygen owing to the consistency of the medium as well as to the presence of reducing substances in the brain tissue. The blackening which occurred from the growth of the proteolytic group he considered to be due to the action of sulphuretted hydrogen on iron in the presence of alkali. All the anaerobes von Hibler studied formed H_2S but the combination with iron only occurred where alkali was also produced. The use of muscle in place of brain was very briefly considered. The proteolytic group turned the muscle greyish or brownish-green to black while the active carbohydrate-fermenting group changed the color to red. Von Hibler further noted that the harmful effects of the acids formed in the meat on the bacteria were much less than those found in milk or the carbohydrate serum media.

Miss Robertson (1916) carefully analyzed von Hibler's results and substituted bullock's heart with equal quantities of water, for the brain in the emulsion, made the medium alkaline to litmus and found it equally useful for differentiation. She noted that the blackening may be delayed or absent if the medium remains acid.

Henry (1917) using the Robertson medium described further useful reactions for distinguishing certain of the anaerobes.

He pointed out that the muscle glycogen is converted into glucose and isomaltose so that the carbohydrate content in this medium approximates one per cent. The production of tyrosin in large amount by *B. histolyticus* in lesser quantities by *B. sporogenes* and most variably by *B. welchii*, are useful observations as indications of protein digestion. The red color change in the meat he considered due to an acid derivative of hemoglobin and according to the amounts of acid produced it is bright and stable or pink and readily decolorized. The blackening of the meat he believed to be due to the formation of iron sulphide but thought it might be a tyrosin derivative or a humin substance from condensation of an amino body with a sugar.

McIntosh (1917) made little or no use of the meat medium and the value of his important contribution to the classification of the anaerobic bacteria of war wounds suffers in consequence.

While working in the Robert Walton Goelet Research Laboratory in the Hospital Complementary V.R. 76 at Ris-Orangis, and later at the American Red Cross Hospital of Paris in 1916-1917, I had experience with a cooked meat medium made from beef muscle in the study of the bacteria of war wounds. Since my return I have found it a most useful medium for the growth of a great variety of bacteria, anaerobic and aerobic.

The medium is made as follows: the fresh meat is freed from fat and gross fibers, finely minced, ground in a mortar and mixed with an equal part of water. It is then slowly heated to boiling with constant stirring to allow the soluble albumins to coagulate about the meat particles. This coagulated albumin serves in itself as a favorable medium for the anaerobes as Tarozi has shown. The emulsion of meat is neutralized or made slightly alkaline, using hot titration with phenolphthalein, tubed at least two inches high and autoclaved at 115°C. for at least half an hour. Just before use, the tubes are put in flowing steam for half an hour and then rapidly cooled. The seeding is done by pipette, swab or needle and the material thoroughly mixed with the meat particles.

There are no further precautions necessary to obtain anaerobic conditions. All the anaerobes from war wounds which I have

studied grow readily in these open tubes such as *B. welchii*, vibriion septique, *B. oedematiens*, *B. tertius*, *B. fallax*, *B. aerofetidus*, *B. sporogenes*, *B. histolyticus*, *B. tetani*, *B. pseudotetani* and others. Not only do the anaerobes grow luxuriantly in this medium but the aerobes do equally well. In the routine study of war wounds I have found the cooked meat the best general medium for the growth of the mixed flora liable to be encountered. Hemolytic and non-hemolytic streptococci, capsulated cocci, staphylococci, Gram negative cocci, various diphtheroids, colon-like bacilli, members of the *B. mucosus-capsulatus* group, *B. proteus*, *B. pyocyaneus* and many other bacteria grow readily. The chain formation of the streptococci is particularly well shown and the morphological differences between the *Streptococcus viridans* and the *Streptococcus hemolyticus* groups are clear and have been confirmed by blood agar plating.

Perhaps the most favorable character of this medium, after its general growth stimulating influence, is that the products of growth do not rapidly destroy the various forms and of all the media in common use it is to the meat medium that one can constantly return to reisolate bacteria which have died out or have become hopelessly overgrown in other media. Just what the exact conditions are which favor this symbiosis is hard to say. The meat particles act no doubt as buffers to the extremes of reaction, especially absorbing undue amounts of the acids produced in the cultures. I have been able to reisolate a great variety of bacteria from the original mixed meat cultures which I brought with me from France. These reisolations from mixed cultures have been made after from six to ten months and it is quite remarkable the number of cases from which I have regrown the same types of organisms as were originally rapidly isolated. I have grown in this way streptococci of the viridans group, staphylococci, Gram negative cocci, diphtheroids and many others besides a great variety of anaerobes. Some of these latter were partially separated and were stored on the meat medium for further study. In no case, of those I have restudied, with two possible exceptions, have I failed to regrow the various anaerobes. The meat is the best single medium we have for

studying the anaerobes of war wounds, the reactions are useful for rapid differentiation of groups as well as for individual identification, and no other medium we have can so readily indicate the presence of anaerobes in mixed cultures where they are often not expected. I have confirmed the changes in the medium described and splendidly illustrated by Henry in my study of anaerobes from war wounds as well as for anaerobes from civilian cases and other sources. A fuller discussion of these will appear in a later paper on anaerobic bacteria.

I have also used this medium for growing other anaerobes with good results. A culture of *Actinomyces* which I isolated from a human case grows well in the open meat medium, in whitish granules which can be readily picked, examined in moist preparations or crushed and reinoculated in glucose agar shakes. The white granules of varying size develop best in a zone similar to that in a glucose agar shake. The growth so obtained makes useful material for student teaching in lieu of the fresh pus. Another anaerobe of interest here is the bacillus of acne which grows rapidly and particularly well in this medium the growth forming heavy white deposits on the meat particles in the upper portions of the tube. For these two anaerobes, which are more or less difficult to store, this medium is most useful. The culture of *Actinomyces* has been transferred after one hundred and fifty days and showed no evidence of dying out and the bacillus of acne is equally long lived and gives the typical ring growth in glucose agar on transfers up to at least five months. Cultures made from the scrapings of the teeth in this medium to indicate one of its many general uses, reveal growths of many leptothrix, fusiform and other microorganisms often seen in direct smear but rarely cultivated.

It is important to remember that in the use of fresh tissue, although this is still necessary for the growths of many of the stricter parasites, there is always present the disturbing possibility of contamination, not so much from without as from the animal tissue itself. This has been pointed out by many workers from Theobald Smith (1890) to the later work of Ford, Tarozzi, Adami, Wolbach and Saiki and many others, It is so uncertain

a factor that fresh tissue in culture media cannot be employed as a method for the routine work for the general bacteriological laboratory and it is just here that the cooked meat medium fills a long felt want.

De Kruif, Adams and Ireland in confirming the results of Bull and Pritchett on the toxin production of *B. welchii* found • the amount of toxin to be as great in a veal mash broth as with fresh sterile tissue.

This meat medium finds in the study of the anaerobes perhaps its most useful place and offers a broad field of study for the biological chemist as well as for the bacteriologist. Investigation of the results of proteolysis by pure cultures of well identified anaerobes has received but little attention. The study of crystal forms which moist preparations show to be abundant in many of these cultures will undoubtedly amply repay the time expended. The application of these studies to the activities of the anaerobes in the intestinal tract, more especially in putrefactive conditions, may well lead to a clarification of many vexed questions.

CONCLUSIONS

The cooked meat medium is the most useful medium we have at present for obtaining growth of both anaerobic and aerobic bacteria, for storing mixed cultures for later isolation as well as pure cultures for further investigations.

We have in this medium a ready means for the cultivation of the anaerobes and the storing of pure cultures. The changes in reaction, color, odor and the evidences of varying degrees of proteolysis make it of the greatest importance in differentiating many of the types and adds to its value. It is further most useful in growing and storing cultures of the *Actinomyces hominis*, *Bacillus acne* and many others.

It is a medium simply made which can be readily sterilized and owing to its high buffer character is adaptable to the growth of a great variety of bacteria.

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THE DELAYED DEVELOPMENT OF COLONIES ON PLATES SEEDED FROM DISINFECTED SEWAGE

W. S. STURGES, JR.

Bacteriologist, Sewage Experiment Station, New Haven, Connecticut

Received for publication September 28, 1918

An experiment station for the study of various methods of treating the sewage of New Haven was operated by the city during the year June 1917 to May 1918, under the direction of a Committee of which Prof. C.-E. A. Winslow of Yale University was Chairman; and the author desires to express to Professor Winslow, and to Prof. L. F. Rettger, his thanks for advice and suggestions in regard to the work reported here. Chemical analyses were made by Dr. F. W. Mohlman, Chemist in charge of the station.

The New Haven Sewage Experiment Station offered peculiar opportunities for observing the effect of three widely used disinfectants—namely, copper, chlorine and sulphurous acid—on the bacterial content of sewage. The first of these substances occurs in the sewage itself in varying amounts during the working hours of the day, being derived from a trade waste discharged into the sewage by munition works at a distance of $2\frac{1}{2}$ miles above the station. During the manufacturing process in question shells are washed in hot water and sulphuric acid. This acid wash containing considerable copper sulphate is discharged into the sewer. The alkalinity of the sewage is sufficient to neutralize this acid waste except in occasional instances. The copper sulphate is probably changed first into the basic carbonate and then gradually converted into the hydroxide. Since the latter is insoluble it is presumably before this last change takes place that the copper exerts its disinfecting action. The other disinfectants discussed, sulphurous acid and chlorine, were added to the sewage as a part of our investigation. The sulphur

dioxide was applied as part of the Miles acid process of treatment in quantities sufficient to keep the effluent faintly acid. The acidified sewage was retained for four hours at the end of which time bacteriological examination was made. Liquid chlorine was added to the effluents of three of the processes under investigation in the usual way, plates being made after a period of contact of thirty minutes.

ANTISEPTIC ACTION OF COPPER SALTS

It was soon apparent, after beginning our investigation, that trade wastes were exerting a strong disinfectant action on the sewage at this outlet. A comparison of the counts made at different hours of the day indicates this fact.

It will be seen from tables 1 and 2 that the early morning and Sunday samples of sewage have very much higher counts than samples taken at hours when factory wastes might be expected to be present.

Evidence as to the exact source of the disinfectant was sought by taking samples at different points along the sewer from its outfall upward along its course. The results are given in table 3.

It will be noted that above the munition works a total count of nearly 1,000,000 bacteria per cubic centimeter and 100,000 gas formers per cubic centimeter was found, while below the munition works the total count was reduced to a few thousand and the gas formers to 100. Above the munition works no copper was detectable while the sewage immediately below contained nearly 9 parts per million. All samples were alkaline. It seems reasonable, therefore, to conclude that the low count of the sewage noticed throughout the week days in our investigation was due to the disinfectant action of this copper waste. This is quite in keeping with the results obtained by Johnson and Copeland¹ on the application of copper as a disinfecting agent for sewage. Some of their results at the Columbus Testing Station are shown below.

¹ Journ. Infect. Dis., Sup. I, 327, 1905.

TABLE 1
Disinfection of sewage by copper, August 10, 1917

TIME	PARTS PER MILLION				DECOLORIZATION TIME METHYLENE BLUE		BACTERIA PER CUBIC CENTIMETER 20 °C.	GAS FORMERS PER CUBIC CENTIMETER 37 °C.
	Chlorine	Alkalinity	Iron	Copper	Undiluted	1 Sewage 2 water		
					hours	hours		
a.m.								
6.00	30	94	0.1	0.56	6	120	3,700,000	100,000
7.00	3,750	136	0.2	1.52	6	24		
8.00	3,100	160	5.0	1.36	24	72	2,500,000	1,000,000
9.00	2,900	101	1.8	2.88	48	120		
10.00	2,800	43	0.3	4.80	168	192	19,000	100
11.00	2,750	114	0.4	2.72	60	144		
12.00	2,800	98	0.2	4.00	144	168	559,000	1,000
p.m.								
1.00	3,075	6	5 5	5.60	240	240		
2.00	3,200	86	0 6	1.92	24	72	187,000	10,000
3.00	3,325	91	0.7	1.60	24	120		
4.00	3,375	88	0.2	1.92	48	96	39,000	100
5.00	3,450	98	0.1	3.52	150	144		
6.00	130	103	0.4	2.88	216	100	45,000	1,000
7.00	95	63	1.4	1.28	72	80		
8.00	52	92	0.2	0.64	24	96	33,000	1,000
9.00	50	96	0.0	0.44	6	24		

TABLE 2
Bacterial content of sewage, July 9-26

DAY	TOTAL COUNT PER CUBIC CENTIMETER, AGAR 20°C.				GAS FORMERS PER CUBIC CENTIMETER, 37°C.			
	8.00 a.m.	10.00 a.m.	1.00 p.m.	4.00 p.m.	8.00 a.m.	10.00 a.m.	1.00 p.m.	4.00 p.m.
Monday...	1,500,000	225,000	59,000	16,000	100,000	1,000	1,000	100
Tuesday...	1,080,000	128,000	5,000	7,500	100,000	100	1,000	100
Wednesday...	1,670,000	81,000	7,000	100,000	100,000	100	100	1,000
Thursday...	1,100,000	148,000	1,500	12,000	100,000	10,000	100	10
Friday....	1,125,000	42,000	9,000	9,000	100,000	100	100	100
Saturday...		68,500	7,100	800,000*		100	10	100,000*
Average....	1,295,000	115,400	14,700	159,000	100,000	1,900	385	17,000
Sunday...		3,355,000	2,275,000	2,535,000		100,000	100,000	100,000

*Factories shut down at noon Saturday.

The period of contact in our case is the length of time that it takes the sewage to flow from the munition works to our laboratory. This we compute to be in the neighborhood of one hour. Our analysis (table 3) showed 8.8 parts of copper per million at the munition works but this is doubtless often exceeded, as we frequently obtained samples with lower bacterial content. It is to be expected therefore that the bacterial content of our sewage might frequently be reduced to a few thousand per cubic centimeter.

TABLE 3
Source of copper in the New Haven sewage

DESCRIPTION OF SAMPLE	NUMBER	PARTS PER MILLION		BACTERIA PER CUBIC CENTIME- TER 20°C.	GAS FORMERS PER CUBIC CENTIME- TERS 37°C.
		Alkalinity	Copper		
Near source of sewer	1	54	0.0	990,000	100,000
Below munitions factory {	2	90	8.8	3,000	100
	3	165	5.6	3,000	1,000
Small branch sewer	4	134	0.0	559,000	10,000
Main sewer {	5			70,000	100
	6	156	1.9	380,000	10,000
	7	90	3.5	65,000	1,000
Experiment station	8	130		53,000	1,000

On noting some of the very low counts obtained it occurred to us that possibly we were not finding all of the organisms that were really present. This proved to be the case. By prolonged incubation a count often developed many times greater than the one obtained at the end of two or three days. This phenomenon was so marked in the case of the "disinfected" samples that it was suspected that they differed in this respect from the samples containing no trade waste.

Accordingly in making counts of subsequent samples of raw sewage plates were kept in the incubator and recounted at the end of seven days. The "disinfected" samples as a rule show a marked increase on prolonged incubation. This increase is

quite variable as doubtless is the amount of copper in each sample. Frequently however the count at the end of seven days shows an increase of several hundred per cent over the three day count. This increase tends to be most marked in the samples having low initial count, that is in samples that have been most completely disinfected.

Space does not permit the full tabulation of all the results obtained. Seventy samples of raw sewage which were collected on Sundays or Saturday afternoons and at 8.00 a.m. on week-days (sewage containing relatively small amounts of copper wastes) gave an average of 222,000 bacteria per cubic centimeter after three days' incubation and an average of 348,000 after seven days' incubation. The per cent increase for individual samples between three and seven days varied from 2 per cent to 478 per cent and averaged 93 per cent. On the other hand 68 samples of sewage collected on week days after 8.00 a.m. (containing large amounts of copper wastes) gave an average count of 85,000 per cubic centimeter after three days and an average count of 155,000 per cubic centimeter after seven days. The per cent increase from the third to the seventh day varied from 17 to 1000 per cent and averaged 203 per cent.

A closer measure of the effect of the copper salts in delaying the development of colonies among the bacteria which they fail to kill outright is obtained by grading the samples of crude sewage according to their actual copper content. This was roughly accomplished as follows.

In determining the non volatile suspended matter in our chemical analyses we ignited the mat in a Gooch crucible after filtering 100 cc. of the sewage. After ignition the residual suspended matter on these mats was often found to be distinctly red, owing no doubt to the presence of cupric oxide. Each sample was placed in one or another of five groups according to the depth of color in the ignited mat. This crude determination probably gives a rough indication of the amount of copper salts present. In table 5 the samples of specially high and specially low copper content are compared upon this basis.

The count for the low copper samples doubled on incubating

for seven days while the seven day count for the samples high in copper was over four times as great as the three day count. The average increase on prolonged incubation was three and one half times greater in the high copper than in the low copper group.

Three explanations of this delayed colony formation on the plates seeded with sewage containing copper salts suggest themselves. In some instances it might be assumed that the copper was present in sufficient strength to exert an inhibitory action in the plates after the diluted sewage has been mixed with the

TABLE 4

Effect of copper sulphate on bacteria in Columbus sewage (Johnson and Copeland, 1904). Composition of sewage

ORGANIC NITROGEN		ALKALINITY		BACTERIA PER CUBIC CENTIMETER
p.p.m.		p.p.m.		
6.3		376		1,200,000

Cu	TOTAL COUNT ON GELATIN AT 20°C.			
	Contact: 0	1 hour	6 hours	24 hours
p.p.m.				
0	1,200,000	1,200,000	6,000,000	14,000,000
5	1,200,000	14,000	700	3,400,000
25	1,200,000	9,500	250	200
250	1,200,000	3,000	190	35

media; although the antiseptic must here be in a very high dilution. If the original sewage sample contained 10 parts per million a plate poured for a dilution of 1:100 would contain about 1 part of copper in 10,000,000 parts of medium. Still it is conceivable that even such a small amount of a strong antiseptic might retard the growth of some of the organisms we are dealing with when they are suddenly placed in such a new environment. A single, isolated organism, especially if of animal origin, might well be retarded a few days by an extremely slight amount of an inhibiting substance. An attempt was made to confirm this view by adding slight amounts of a copper salt solution to a sample of Sunday sewage at the time of plating.

TABLE 5

Three and seven day counts of raw sewage containing various amounts of copper

LOW COPPER CONTENT—BACTERIA PER CUBIC CENTIMETER			HIGH COPPER CONTENT—BACTERIA PER CUBIC CENTIMETER		
3-Day count	7-Day count	Percentage increase	3-Day count	7-Day count	Percentage increase
144,000	269,000	87	245,000	395,000	20
120,000	244,000	104	23,000	98,000	330
160,000	312,000	95	9,000	53,000	490
132,000	277,000	110	33,000	73,000	121
126,000	270,000	114	41,000	150,000	266
134,000	396,000	200	127,000	252,000	99
142,000	340,000	140	7,000	43,000	510
245,000	395,000	61	3,000	15,000	400
300,000	465,000	55	10,000	47,000	370
70,000	175,000	150	185,000	320,000	73
36,000	117,000	225	3,000	12,000	300
65,000	135,000	110	3,000	33,000	1000
230,000	500,000	115	3,000	20,000	566
34,000	196,000	480			
115,000	170,000	48			
110,000	360,000	240			
168,000	285,000	70			
375,000	660,000	76			
250,000	530,000	112			
290,000	420,000	50			
154,000	183,000	18			
7,000	7,500	7			
150,000	355,000	136			
21,000	29,000	40			
177,000	292,000	65			
50,000	60,000	20			
152,000	245,000	62			
190,000	255,000	34			
98,000	228,000	133			
23,000	28,000	22			
205,000	340,000	66			
154,000	249,000	63			
250,000	445,000	78			
165,000	280,000	70			
Average increase for low copper samples		101	Average increase for high copper samples		349

Counts were made at the end of three and seven days incubation but neither 5, 10 nor 20 parts of copper caused any appreciable increase in the difference between the first and second count.

The second explanation that suggests itself is that prior to plating some of the organisms have been injured, so to speak, by the copper so that it takes them a few days to recover. They are not killed but are in such a weakened condition that immediate growth is impossible. A purely hypothetical explanation of this lag period is that it is necessary for the previously absorbed antiseptic to diffuse out of the organisms before growth will take place.

A third possibility is that the organisms which survive the copper treatment may be of slowly growing types. We thought at first that the presence of a large proportion of spore formers might account for the phenomenon but a few heating tests failed to show a specially high proportion of spore bearing organisms on the seven day plates.

ANTISEPTIC ACTION OF SULPHUROUS ACID AND SULPHITES

In the Miles' acid process of sewage treatment we added sulphur dioxide in sufficient quantities to neutralize the alkalinity of the sewage and keep the effluent constantly acid. The sewage with a free acidity of 50 to 100 parts per million (expressed as calcium carbonate) was retained in a sedimentation tank for four hours. Plates of the effluent were then poured and counts made after three and seven days incubation. As may be seen from table 6 a very marked lag period was noted on the plates seeded with this effluent.

While a two or three day count is usually remarkably low this is therefore by no means a correct measure of the number of living organisms present in the Miles' effluent. Prolonged incubation will often indicate a total count of ten times this magnitude. The average of the percentage increase of the total counts between three and seven days incubation is 560 per cent. Such delayed growth should be taken into consideration in any bac-

teriological examination of this type of sewage effluent. It is easy to imagine that the bacterial removal is greater than it really is. On the other hand the bacterial efficiency of the

TABLE 6

Three and seven day counts of Miles' acid effluents showing the delayed development due to sulphurous acid

3-DAY COUNT	7-DAY COUNT	PERCENTAGE INCREASE	3-DAY COUNT	7-DAY COUNT	PERCENTAGE INCREASE
620	2,290	269	22,000	71,000	220
8,000	22,200	178	2,570	9,000	250
700	1,510	116	1,100	3,300	200
900	7,600	744	4,850	13,000	170
5,400	12,000	122	200	1,900	850
6,400	20,000	212	200	2,800	1300
1,240	9,200	642	80	190	140
31,000	42,000	35	245	3,200	1200
15,000	40,000	166	1,000	4,700	370
2,500	19,300	672	2,000	7,000	250
1,700	7,000	316	200	1,000	400
590	8,000	1,250	30	90	200
2,150	11,000	411	30	60	100
42,800	110,000	157	150	1,400	830
32,000	100,000	210	40	360	800
268	1,800	570	10	600	5900
1,140	4,185	270	1,800	11,000	510
3,800	14,400	280	41	96	130
2,760	9,500	240	50	1,600	3100
430	1,100	160	35	350	900
3,200	8,300	160	105	805	670
1,000	9,000	800	400	1,100	170
460	3,920	750	233	1,600	590
3,800	14,400	280	600	1,600	170
20	140	600	130	390	200
570	3,350	490	1,140	3,200	180
20,000	44,000	120	470	2,580	450
2,100	7,100	240	360	1,500	320
6,500	20,000	210	535	4,400	720
860	4,500	420	225	3,300	1370

Average Increase, 560 per cent

Miles process is so high that even the number of colonies obtained after seven days incubation is but small and there may be no sanitary significance to the organisms that develop after

three days incubation. It seems probable from our low *B. coli* counts that the pathogens are practically eliminated. It was not possible in our work to do anything in the way of classifying these organisms showing delayed growth except to find, as noted above, that they were mostly non-spore formers.

To find the number of organisms per cubic centimeter in this low count effluent it is necessary to pour plates with a dilution of only 1 to 10. In this low dilution the free sulphurous acid might be present to the extent of one part per million of media, besides which there are considerable amounts of sulphites present. This is doubtless sufficient to exert an antiseptic action in the plates during incubation. Some better method of getting a total count is highly desirable. Trials were made in which the effluent was neutralized before plating; but the antiseptic action was still very strong, probably due to the presence of sulphites.

ANTISEPTIC ACTION OF CHLORINE

Finally, in connection with our study of the effect of varying amounts of chlorine on the bacterial content of raw sewage, plates were poured immediately before chlorinating and after being exposed to chlorine for one half hour. The plates of some of these chlorinated samples, with those of the corresponding raw samples, were incubated for seven days and the count then compared with the three day count. 6 and 8 parts per million of liquid chlorine were employed. The results are shown in table 7.

The results of this limited number of observations appear rather erratic. It is evident, however, that there is no increase in the seven day over the three day count to compare with that in the case of samples disinfected by sulphurous acid. In general there is no very marked difference between the chlorinated and the corresponding raw samples. In 16 out of the 24 cases there was a slightly greater increase in the case of the chlorinated samples while in the other 8 cases the increase was actually less in the case of the chlorinated samples. If we take the averages we find that the increase for the chlorinated is about

the same as that for the unchlorinated samples. If we were justified in drawing any conclusion from this table it would be that chlorine when used in amounts sufficient to have a very

TABLE 7

Three and seven day counts of chlorinated and unchlorinated samples of raw sewage

CHLORINATED			CORRESPONDING RAW		
3-Day	7-Day	Percentage increase	3-Day	7-Day	Percentage increase
16,600	23,500	42	380,000	384,000	1
500	500	0	9,600	18,000	88
3,000	12,000	300	138,000	162,000	17
3,340	7,000	110	55,000	80,000	45
1,000	3,200	220	22,000	116,000	430
1,300	2,300	77	224,000	340,000	52
8,000	18,000	13	18,000	38,000	111
16,000	24,000	50	175,000	243,000	39
1,250	3,200	156	66,000	95,000	44
4,000	10,000	150	127,000	252,000	98
2,000	14,000	600	157,000	287,000	83
2,600	6,400	146	390,000	800,000	105
700	1,300	86	10,000	28,000	180
1,350	2,650	96	4,000	15,000	275
90,000	146,000	62	380,000	605,000	59
106,000	180,000	70	720,000	1,060,000	47
3,000	4,400	47	320,000	340,000	6
580	710	22	17,000	31,500	85
1,500	1,600	7	141,000	225,000	60
820	2,100	156	160,000	300,000	88
1,900	3,500	84	119,000	165,000	39
2,800	4,400	57	82,000	120,000	46
1,000	3,500	250	4,900	16,000	227
125	125	0	15,000	41,000	173
Average increase chlorinated		115	Average increase unchlorinated		100

strong bactericidal action (90 per cent removal or better) has little or no tendency to cause delayed colony formation among the surviving organisms.

SUMMARY

If copper salts are added to sewage in sufficient quantities to effect considerable bacterial reduction there is a marked tendency to delayed colony formation among the surviving organisms.

In previous investigations such as that of Johnson and Cope-land some of the "disinfectant" action observed was doubtless merely due to this property.

Sulphur dioxide when used to disinfect sewage has an even greater tendency to produce delayed colony formation than have copper salts.

This phenomenon interferes with the common method of determining the count. The actual bacterial content of a Miles' acid effluent may readily be ten times as great as a two or three day 20° count.

Such evidence as has been obtained suggests that chlorine does not produce delayed colony formation of the type indicated.

The delay in colony formation is ^{of} either:

a. To the antiseptic action of ^{of} ~~mix~~ ^{use} amounts of copper or sulphurous acid or sulphites carried over in the dilution process into the plates. This action is probably important in the case of the sulphurous acids and bisulphites where plates were made at only a 1 to 10 dilution. It is probably relatively slight in the case of the copper with plates poured after 1 to 100 dilution and containing only 1 part of copper in 10,000,000 parts of medium.

b. To a selective action of the disinfectants which may perhaps kill out quickly growing and spare slowly growing types. As throwing light upon this possibility it should be noted that no evidence was found of an increased proportion of spore bearers among the slowly growing forms.

c. To a possible lowering of the vitality of the organisms affected by the disinfectant so that while not killed outright they are able to develop only slowly and after an appreciable lag period when transplanted to a favorable medium.

The completion of the operating period planned for the experiment station prevented a further study of these various possi-

bilities. The results so far obtained are presented here in view of their practical bearing on the period of incubation to be adopted for plates seeded with certain types of disinfected effluents and because of the theoretical interest of the possibility that the slow development of colonies on plates made from such effluents is due to a lowering of vitality produced by a sublethal dose of disinfectant.

METHODS FOR THE ISOLATION AND CULTIVATION OF *BACILLUS PUTRIFICUS* AND OTHER OBLIGATE ANAEROBES

WM. S. STURGES, JR. AND LEO F. RETTGER

From the Sheffield Laboratory of Bacteriology and Hygiene, Yale University

Received for publication October 9, 1918

Bacillus putrificus is an almost ubiquitous putrefactive anaerobe which has received comparatively little study. Its important rôle in the natural decomposition of protein material was discovered by Bienstock (1899 and 1901) who has given us a graphic description of this, as yet more or less obscure, organism. Bienstock's observations were in the main substantiated by Rettger (1906, 1908). On account of its somewhat close resemblance to *B. tetani*, particularly in its morphology and certain cultural requirements, *B. putrificus* has often been mistaken for the tetanus bacillus. From this standpoint alone a wider knowledge of the important properties of *B. putrificus* seems desirable.

Although the Bienstock bacillus is so widely distributed in nature, it soon becomes apparent to any one who attempts to isolate it from its natural sources that this process is extremely difficult. Repeated efforts in this laboratory to effect a complete isolation by methods heretofore in vogue for anaerobes have always failed. Colonies seldom develop in the Novy jar, and when they do develop they are found to be impure. The tendency of this organism to grow into colony form only when it is associated with some other organism which favors its development is very marked, whereas *B. oedematis* and *B. chauwei* (*anthracis-symptomatici*), its close allies in the processes of protein decomposition, will often under similar conditions produce characteristic and pure colonies.

The organisms which are usually associated with *B. putrificus* in attempts at isolation are found to be spore formers, either aerobes or anaerobes. It is impossible, therefore, to eliminate them, by the heat method. Re-plating likewise proves unsuccessful. By replacing these associated organisms with known non-spore-bearing forms, however, a method was soon devised of isolating *B. putrificus*. *B. coli* was first employed, and after, definite periods of associated growth it was destroyed by heating the mixture at 80°C. for fifteen minutes.

Tubes of liquid agar were inoculated with putrefying material known to contain *B. putrificus*, and with varying amounts of *B. coli* cultures, and poured into Petri dishes. The plates were incubated in the Novy jar. Some of the colonies which developed were found, by microscopic examination, to be made up of *B. putrificus* and *B. coli*. Subculture from such colonies into eggmeat medium, and heating at 80°C., yielded pure cultures of the putrificus bacillus.

The following modification of the above method of isolating obligate anaerobes has greatly simplified it without lessening its efficiency. By substituting *Staphylococcus aureus* for *B. coli*, symbiotic growths (mixed colonies) were readily obtained by the usual method of open or aerobic culture. The amounts of the putrefying material and of the staphylococcus suspension were varied in order to obtain the most favorable balance of the two in the culture plates. After incubation at 37° for three or four days some of the irregularly-shaped yellow colonies were subcultured in egg-meat medium, in which putrefaction and protein liquefaction occurred after incubation for a few days at 37°C. Subsequent heating at 80° destroyed the staphylococcus, leaving the putrificus in pure form.

Five isolations were effected by the above improved method.

A SECOND METHOD

In a small number of our putrefying mixtures *B. putrificus* appeared to preponderate over the other organisms. This preponderance in numbers was easily observed in stained prepara-

tions. For such bacterial mixtures the following plan was evolved.

A loopful of the material is suspended in sterile water and well shaken. A direct count is then made, of the morphologically characteristic putrificus spores per cubic centimeter of the diluted medium, irrespective of all other forms. The bacterial suspension is further diluted with water until it contains from 10 to 50 of these spores to the cubic centimeter. Amounts of this diluted material varying from one loopful to 0.1 cc. are introduced into a series of egg-meat medium tubes. After inoculation with *Staphylococcus* the egg-meat tubes are incubated at 37°C. for a week. Of these a few will as a rule contain only *B. putrificus* and the *Staphylococcus*, which upon heating will yield pure cultures of the anaerobe in question. It is to be noted that this procedure is especially applicable when the original material contains a relatively large number of *B. putrificus* spores.

Three isolations were effected by this method.

CULTIVATION OF OBLIGATE ANAEROBES

As has been intimated, a very satisfactory way of cultivating anaerobes after they are once isolated is by symbiosis with some aerobic non-sporing organism under so-called aerobic conditions, or more correctly, by the usual aerobic methods. All of the aerobes employed by us proved equally effective in this connection. Tubes of egg-meat medium thus inoculated (*B. putrificus* and an aerobe) undergo vigorous putrefaction in the course of a few days, usually within three or four. Whenever the anaerobe is desired in pure culture heating at 80°C. readily removes the associated organisms. The most satisfactory medium for growing and maintaining putrefying organisms was found to be the egg-meat medium previously described by Rettger (1906). In this medium the putrefying anaerobes develop very rapidly and their putrefactive properties are well displayed. They remain viable for practically indefinite periods of time. Old tubes which had been kept in this laboratory for

at least two years readily yielded characteristic growths on transplantation to a new medium.

All of the strains of *B. putrificus* isolated by us exhibit a peculiar reluctance in undergoing development and in attacking the egg-meat medium in pure culture. The putrefaction is very much delayed, and does not begin as a rule until twenty to thirty days after the beginning of incubation under anaerobic conditions. When once begun, however, the putrefaction is rapid and typical. This is in striking contrast with the other well-known putrefyers (*B. oedematis* and *B. chauvei*) which usually begin to decompose the protein within a period of three or four days. This delayed putrefaction of *B. putrificus* occurs only in pure cultures. When growing in symbiosis with another organism, preferably an aerobe, it very quickly assumes a putrefactive rôle. The nature of this symbiosis is now under investigation. It will be of special interest to determine whether the associated organism favors putrefaction merely by rendering the conditions strictly anaerobic, or whether there are intermediary products of metabolism of the aerobes which favor the development of the obligate anaerobe.

It is quite apparent from a review of the literature that previous investigators have not been working with pure cultures of *B. putrificus*, as this phenomenon of delayed putrefaction which appears to be a constant feature of pure cultures of the organism has not been pointed out by them. We have attempted at different times to obtain pure cultures from other laboratories. The few cultures which were acquired in this way were found to contain other organisms.

The possible application of these methods to the isolation and cultivation of other fastidious anaerobes, particularly *B. tetani* and *B. welchii*, presents an interesting subject for investigation. Other media would be required, however, in place of the egg-meat mixture. Experience with the tetanus bacillus has shown how difficult it is to obtain this organism in pure culture, and it is not improbable that many of the so-called pure cultures of *B. tetani* are mixtures of this anaerobe and some other organism which is highly favorable to the growth and perhaps even to the toxin production of the tetanus bacillus.

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A PRELIMINARY REPORT UPON SOME HALOPHILIC BACTERIA

EDWIN LEFEVRE AND LESTER A. ROUND

Bureau of Chemistry, United States Department of Agriculture, Washington, D. C.

Received for publication October 9, 1918

While studying the microorganisms concerned in the fermentation of cucumber pickles a group of bacteria were isolated and studied which appear sufficiently interesting to deserve special comment. These organisms were isolated from the scum which grows on the surface of the brine after active fermentation (respiration) has ceased. The interesting fact about these organisms is that certain members of the group demand an appreciable concentration of sodium chloride for development.

HISTORICAL

Many investigators, among others Forster (1889), de Freytag (1890), Petri (1890) and Peuch (1887) have studied the salt tolerance of some of the well-known species of pathogenic bacteria such as *B. tuberculosis*, *B. typhosus*, *B. diphtheriae*, *Vibrio cholerae*, *B. anthracis*, and the bacillus of swine plague (Schweinrotlauf). Many of these types were found surprisingly resistant, being still living and virulent after months of exposure to saturated salt solutions. Sperlich (1912) studied the reaction of bacteria found in air, water and soil to varying percentages of salt. He found that many organisms grew better in the presence of a certain percentage of salt, the optimum varying from 0.5 per cent to 5 to 6 per cent of salt in the case of *Bact. constrictum* (Zimmermann).

Farlow (1867, 1886), Poulsen (1879-1880), Edington (1887), Leyet (1887), Ewart (1887), Le Dantec (1891), Hoyer (1901, 1904, 1906, 1908), Beckwith (1911), Kellermann (1914-1915)

and Bitting (1911), have studied the bacteria concerned in the reddening of salted fish. The bacteriology of this phenomenon seems to have been fairly well worked out. Kellermann speaks of other non-pigment-forming bacteria associated with the causative agent but none of these investigators have called attention to a group of bacteria that demand for growth a certain percentage of sodium chloride. In this respect we believe the cucumber brine organisms to be unique.

Rapin and Grosseron (1914) have studied the possibilities of bacterial contamination of salt during the process of refining and Wolff (1914) made a study of highly refined salt used for butter making and of a less highly refined grade used for cheese making. Both investigators found that the more highly refined the salt the smaller the number of microorganisms present. Wolff showed that many of the bacteria found had a very marked effect upon butter when applied in pure culture to sterile material.

EXPERIMENTAL

From the pickle scum mentioned above 21 bacilli were isolated. The brine from which the scum was taken contained approximately 10 per cent of salt. The medium used for isolation was cucumber juice agar to which 10 per cent of sodium chloride had been added. On this medium at room temperature there appeared first a large number of rugose, cretaceous, raised colonies of mycoderma, in appearance quite characteristic of the scum as it appears on the surface of the pickle vats. After a few days smaller, creamy, glistening, colonies appeared, 1 to 2 mm. in diameter. These colonies were raised, opaque, with entire edge and of a butyrous consistency. Many of the colonies when viewed from the bottom of the plate had a very bright red center with a dark green periphery. When transferred to cucumber agar slants with ten per cent salt no further chromogenic action was noted except, in some cases, a browning of the medium underneath the stroke. It was found that these organisms would grow apparently just as well on meat extract medium with 5 per cent salt as on the cucumber medium and so for

further transfer of stock cultures and for differential purposes meat extract medium with 5 and 10 per cent salt was used. A study of the cultural characteristics of the organisms divided them into five groups as follows:

Group 1. Non-motile, non-spore-bearing bacilli, average size 0.5 by 2μ . On salt agar smooth, opaque, white, glistening colonies after forty-eight hours at 30°C .

Group 2. Non-motile, non-spore-bearing, Gram-negative bacilli 0.5 by 4 to 6μ . Abundant opaque, glistening colonies on salt agar. Motility doubtful.

Group 3. Gram-positive bacilli 0.5 by 4μ showing elliptical terminal spores. On salt agar plates there were formed at first large myceloid colonies which did not reappear in subcultures.

Group 4. Short, non-spore-forming, Gram-positive, average size 0.4 by 3μ with a tumbling form of motility. Colonies on salt agar convex, round, glistening, butyrous. No spores.

Group 5. Small, motile, non-spore-forming, Gram-positive bacilli 0.3 by 2μ . Colony formation not uniform.

Other cultural characteristics are shown in the following table.

The most conspicuous feature about these organisms is their ability to grow in highly concentrated salt solutions, the majority growing readily in a 25 per cent solution of sodium chloride.¹ In only one of the groups (no. 1), could growths be obtained without the aid of added salt. The optimum temperature was 30°C . But under the most favorable conditions growth was slow. The organisms were very sensitive to acids. Inoculated into pickle juice with varying degrees of acidity, all failed to grow above + 1. All except 3 cultures grew in salt broth with a P_{H} value of 6. In broth with a P_{H} value of 5, growth occurred in but one culture.²

All the cultures were facultative, although growth was more abundant in the presence of air.

No indol was formed and nitrates were reduced by but one of the groups (group 5).

¹ Medium containing 25 grams of sodium chloride in 100 cc. This method was followed in making all the media containing salt.

² The Colorimetric Determinations of Hydrogen Ion Concentration and Its Application in Bacteriology by Clark and Lubs, Journ. Bact., 2, nos. 1, 2 and 3.

Table showing cultural characteristics of five groups of *Halophilic organisms*

GROUP NUMBER	GRAM STAIN	LIQUEFACTION OF GELATIN	ACTION IN MILK	GROWTH ON POTATO	INDOL PRODUCTION	NITRATE PRODUCTION	ACID PRODUCTION* IN			SPECIAL CHARACTERISTICS	RELATION TO SALT	
							Glucose	Lactose	Sucrose.		Minimum requirement	Maximum requirement
											per cent	per cent
1	0	0	No change in plain milk after 20 days but milk with salt added coagulated in 18 days	Moderate raised, glistening	0	0	0	0	0	Decided ropiness in sugar broths	0.0†	15
2	0	0	Coagulated in 5 to 10 days	Raised, spreading, glistening	0	0	0	0	0	Odor of butyric acid in sugar media	5	25
3	+	+	Coagulated in 5 days	Moderate, raised, spreading	0	0	+	0	+	Spore formation	5	25
4	+	0	Not coagulated	Membranous, flat, glistening	0	0	+	0	0	Characteristic growth on potato resembling a coating of shellac	5	25
5	0	0	Not coagulated	Moderate, smooth and glistening	0	+	+	0	0	Cultural characteristics not uniform as in other groups	5	25

* If medium titrated less than 1 per cent normal acid to phenolphthalein, it was recorded as a negative fermentation.

† Ordinary nutrient broth containing 0.5 per cent sodium chloride.

These organisms show great resistance to drying. Thoroughly dried out cultures kept in the laboratory for one year were easily revived by adding salt broth to the tubes.

The form of growth shown in salt broth by most of these cultures was quite characteristic. In this medium there was slowly formed a moderate, uniform cloudiness with a light flaky pellicle, which later became thick and creamy. After several days' incubation, the pellicle broke up, forming viscid, stringy material suspended horizontally in the medium. This settled later and formed a viscid mass at the bottom. In group 1 marked ropiness of the liquid media occurred.

While the tolerance of certain organisms for high concentrations of sodium chloride and the favorable influence of certain concentrations upon growth has been determined for many organisms, no one, so far as the writers are aware, has called attention to a group that absolutely demand sodium chloride for their development.

Investigations have indicated that the chlorides are not essential to the growth and development of bacteria and experience has shown that the presence of salt in more than small percentages is inhibitory to the growth of many types (Sperlich, 1912). All these organisms showed a marked preference for salt and all except group 1 apparently demanded a certain concentration, 2 per cent, for their growth.

No explanation of this phenomenon is offered but the inquiry suggests itself as to whether they are essentially salt organisms which by long environmental influences have come to require salt in high concentration as essential to their vital processes, such as we might expect to find in sea water, or whether they are only common saprophytic bacteria which by growth for only a short time in salt solution have developed mutants which demand salt for their growth.

The question of the influence of osmotic pressure upon these organisms has not been studied. There is the possibility that the essential factor is not sodium chloride but a certain osmotic pressure which is necessary to create a suitable environment for growth. If it is only an increase in osmotic pressure that is

demanded, other salts in proper concentration would act in the same manner as sodium chloride. Further work on this point is contemplated.

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YEAST AUTOLYSATE AS A CULTURE MEDIUM FOR BACTERIA

I. J. KLIGLER

From the Laboratories of the Rockefeller Institute for Medical Research

Received for publication November 23, 1918

The necessity for conserving meat and meat products during the war has rendered a search for cheaper sources of nutritive media for bacteria highly desirable. Douglas and Gordon in England, and more recently Meyer in this country, have proposed the use of peptic and tryptic digests of animal tissues as a substitute for meat extracts and peptones. These materials cheapen the cost of media but necessitate the use of a digestive enzyme, usually varying in potency, and of considerable amounts of valuable animal protein. It seemed that some non-animal protein might prove equally satisfactory.

Yeast naturally suggested itself as a possible substitute. Extracts of yeast have been used in the past to reinforce peptone culture media. Furthermore, yeast readily undergoes autolysis, in the process of which there are formed varying proportions of so-called peptones and aminoacids, while the vitamins remain intact. Thus yeast autolysate may be expected to contain all the nitrogenous elements required for the growth of bacteria. However, the proportions of the individual nitrogenous elements vary with the conditions and duration of the autolysis. The extent of the influence which such variations may have on the growth of bacteria cannot be predicted on theoretical grounds. Hence it became necessary to search empirically for the conditions required to obtain from yeast a satisfactory medium for bacteria.

METHOD OF PREPARATION

In addition to its cheapness the yeast medium is, if anything, easier to prepare than meat extract or meat infusion media.

NUMBER OF EXPERIMENT	COMPOSITION OF MIXTURE	INITIAL P_H	TEMP., NUMBER OF TUBES, NUMBER OF DAYS, NUMBER OF TIMES OF INCUBATION	CHEMICAL TESTS				BACTERIOLOGICAL TESTS			BACTERIOLOGICAL TESTS						TOXIN		
				P_H	Nitrogen content per 100 cc.		P/O ₂ per 100 cc.	Ash per 100 cc.	Broth			Broth						B. diptheriae	B. dysenteriae
					Total	Amino			Str.	Pept.	Muc.	Typhlo- nus	Dys. F.	Dys. Sh.	Diph.	Staph.	Indol B. Coli		
1	200 cc. crude brewers yeast to 1000 cc. distilled water, 30 cc. M/15 NaH ₂ PO ₄ , 10 cc. chloroform; incubated at 37°C.	5.0	1 2 3 5	1 2 3 5	6.10 0.0818	0.0429			+++	++		+++	+++	+++	+++	+++	+		
					0.1128	0.0617			++	+		+++	+++	+++	+++	+++	++		
					6.10 0.0991	0.0566			+++	+		+++	+++	+++	+++	+++	++		
2	Same mixture	6.0	1 2 3 5	1 2 3 5	6.50 0.0930	0.0492			+++	++		+++	+++	+++	+++	+++	+	Not tested	Not tested
					0.1184	0.0662			+++			+++	+++	+++	+++	+++	+		
					6.50 0.1103	0.0657			+	++		+++	+++	+++	+++	+++	+		
3	Same mixture	7.0	1 2 3 5	1 2 3 5	7.30 0.0846	0.0455			+++	+++		+++	+++	+++	+++	+++	+		
					0.1243	0.0665			+++	+++		+++	+++	+++	+++	+++	+		
					7.60 0.1159	0.0640			+++	+++		+++	+++	+++	+++	+++	+		
4	400 cc. crude brewers yeast, 1000 cc. distilled water, 2 grams NaH ₂ PO ₄ , 10 cc. chloroform; incubated at 37°C.	6.1	1 4	1 4	6.10 0.348	0.1803			+++	+		+++	+++	+++	+++	+++	+	Filtrate 10 day broth culture inoculated intraperitoneally into guinea pigs (1) 0.3 cc., no effect (2) 0.6 cc., died 3d day (3) 1.0 cc., died 2nd day	2cc. filtrate 4 day broth culture in ear vein of rabbit; paralysis 48 hours. Died in seventy-two hours.
5	Same mixture	6.5	1 4	1 4	6.50 0.379	0.2078			++	-		+++	+++	+++	+++	+++	+	Same as above (1) 0.3—no effect (2) 0.6—no effect (3) 1.0—no effect	Same amount as above; no effect.
6	150 grams drained yeast, 1000 cc. distilled water, 2 grams NaH ₂ PO ₄ , 5 cc. chloroform; incubated at 37°C.	6.1	1 3	1 3	6.10 0.2520	0.1760	0.1170	0.384	++	+		+++	+++	+++	+++	+++	+	1.0 cc. filtrate of 10 day culture inoculated into each of 2 pigs. Recovered	
7	200 grams drained yeast, rest same as no. 6	6.1	1 3	1 3	6.10 0.2892	0.1922	0.1688	0.485	+++	++		+++	+++	+++	+++	+++	+	Same as above 1 died in 2 days 2 died in 4 days	
8	200 grams drained yeast, 1000 cc. distilled water, 2 grams NaH ₂ PO ₄ , 5 cc. chloroform	6.1	1 24	1 24	6.10 0.2003	0.1314			+++	++		+++	+++	+++	+++	+++			Not tested
9	400 grams drained yeast, 1000 cc. distilled water, 2 grams NaH ₂ PO ₄ ; adjusted to P_H and autoclaved	7.0	1	1	0.128	0.0340			-	-		+++	+++	+++	+++	+++			
10	Nos. 8 and 9 mixed before broth was prepared	10			0.2242	0.0898													
11	Broth, Fairchild peptone				0.3663	0.0522			+++	+++		+++	+++	+++	+++	+++		+	+
12	Veal broth, Witte peptone (Meyer and Stickel)				0.3472	0.0350	0.2	0.4088				+++	+++	+++	+++	+++	+		

Two hundred grams of drained or centrifuged brewer's yeast are suspended in a liter of water, 2 grams of NaH_2PO_4 are added as buffer and the reaction adjusted by the addition of N/NaOH , to P_{H} 6.1. Then 5 cc. chloroform are added and the mixture is thoroughly shaken and incubated at 37°C . for two days. It is necessary to shake the flask occasionally during the incubation, to maintain sterility.

At the end of the incubation period the reaction is brought to P_{H} 7.4 and the autolysate heated in a water bath or in the Arnold for thirty minutes. It is then filtered through paper, tubed and autoclaved.

Agar is prepared by adding 15 grams of agar directly to 1 liter of the unfiltered autolysate, and stirring thoroughly to immerse and soften the agar shreds or powder. The mixture is then heated on the free flame or in the autoclave until the agar is completely dissolved. The reaction is then adjusted to P_{H} 7.4, the mixture is heated on a water bath or in an Arnold for half an hour and the partially clear supernatant fluid is decanted to an Erlenmeyer flask or other vessel. The agar is then cooled and whole egg added to clear. The medium is finally steamed in the Arnold for one half to three quarters of an hour, filtered, tubed and autoclaved.

Special media may be prepared with yeast broth or yeast agar as a base according to the usual procedure.

RESULTS

Table 1 contains a summary of the experimental data on which this report is based. About 300 cc. of crude brewer's yeast or 200 grams of the drained material are sufficient to make 1 liter of broth, the total nitrogen and ash of which are approximately equal to, while the amino N is approximately four to five times as high as that of ordinary broth. The hydrogen concentration at which autolysis is allowed to proceed is an important factor in determining the end products and the suitability of the filtrate for culture media. The optimum concentration is P_{H} 6.1.

Brewer's yeast was used in all the experiments reported in this paper. Both the crude material and the drained and centrifuged yeast were tested. Comparative tests were also made to determine the effect of heat or filtration on the efficacy of the medium. Chemical analyses of the total nitrogen and amino acid content of the autolysate media were made to parallel the cultural tests. The presence of tryptophane was determined by the ability of *B. coli* to produce indol. Toxin production in the case of *B. dysenteriae* and *B. diphtheriae* was tested by animal inoculation.

As a result of these experiments it may be concluded, tentatively at least, that yeast autolysate furnishes a suitable medium for the cultivation of all the common bacteria. The various members of the colon typhoid group thrive better in this medium than in ordinary broth. *B. coli* produces an abundance of indol in twenty-four to forty-eight hours. The Shiga type of *B. dysenteriae* on incubation of three to four days at 37°C. yields a toxin which produces the typical reaction in rabbits—paralysis after forty-eight hours, with typical intestinal lesions. The streptococcus and staphylococcus grow readily, but the pneumococcus does not thrive on the yeast autolysate media. The *B. diphtheriae* grows as well on these media as on ordinary agar or in broth. In the yeast broth it produces toxin, but apparently not of as high a potency as in some peptone broths.

It is not possible at present to predict the extent of the usefulness of yeast autolysate in culture media. Preliminary tests indicate that it can be used as a base for the various special media. Endo and brilliant green media prepared with yeast agar give entirely satisfactory differentiation.

SUMMARY AND CONCLUSIONS

Experiments are presented indicating that yeast permitted to undergo autolysis may serve as a cheap substitute for more expensive animal proteins or their digestion products. The autolysate contains a high percentage of amino nitrogen and a relatively small amount of the higher nitrogen complexes. The

fact that some bacteria, notably pneumococcus, meningococcus, etc., do not thrive as well in the yeast broth as they do in beef infusion media, would indicate that the higher nitrogen complexes—polypeptides, etc.—play some part in bacterial nutrition. On the whole, however, it seems that the yeast autolysate media are entirely satisfactory for the cultivation of the less delicate pathogenic and saprophytic bacteria. Endo and brilliant green plates made with this medium give entirely satisfactory results.

STUDIES IN THE METABOLISM OF ACTINOMYCETES

SELMAN A. WAKSMAN

From the Department of Soil Bacteriology, New Jersey Agricultural Experiment Station

Received for publication December 2, 1918

PART I

I. INTRODUCTORY

The Actinomycetes form an extensive and widely distributed group of microorganisms. Like the bacteria and the molds, they occur in nature both as saprophytes and as parasites of plants and animals. While the other two groups of microorganisms have received much attention, this group has been studied very little either morphologically or physiologically, so that it has not even been decided as yet whether its members should be classified with the bacteria, the fungi, or placed in a class by themselves. Although certain *Actinomyces* species have been known for a long time, it is only within very recent years that a thorough study has been made of the structural and physiological characters of the groups, suggestions presented as to the best methods of classification and attempts made to interpret their probable part in nature.

A complete historical review of the pathogenic forms can be found in the work of Musgrave, Clegg and Polk (1908), while the occurrence and activities of the saprophytes have been reviewed by Krainsky (1914), Waksman and Curtis (1916, 1918), and others.

The growth of the Actinomycetes on organic media is not characteristic and this was the reason why the older workers, limiting themselves only to cultural studies and superficial observations, reported very few species of Actinomycetes to be present in nature. The work of more recent investigators in which differentiation is based on the size and shape of spores

and colonies and pigment production, as by Krainsky (1914) or on the liquefaction of gelatin, size of spores and spiral production, as by Waksman and Curtis (1916), marks a step in advance from that of Beijerinck, Sanfelice, Rossi-Doria, Münter and others, who based their differentiation only on pigment production and cultural characters upon media not standard in composition.

The use of inorganic media for the study of this group of organisms marks another important stage in the proper differentiation and classification of these organisms. Several excellent media of standard composition have been suggested and used very successfully: calcium malate agar¹ and glucose agar used by Krainsky (1914); Czapek's agar by Waksman and Curtis (1916) and malate-glycerin agar by Conn (1917). These media are well adapted for the purpose of bringing out the characteristic macroscopic features of the organisms and also for morphological studies. But one should not limit himself merely to the study of morphological and cultural characters. The morphologist always comes first in the study of a new organism, but the physiologist soon follows and the facts brought out by the latter are of as great and often greater importance in supplying information concerning the nature of the organisms than the facts brought out by the former.

The Actinomycetes, like other living things, exhibit phenomena of variability often neglected by the students of microscopic forms of life. The species described are not absolutely fixed, either physiologically or morphologically. A certain physiological feature will depend not only on the species and the

¹ The composition of these media is as follows:

Ca-Malate agar: Water 1000 cc, agar 15 grams, Ca-malate 10 grams 1 per cent, NH_4Cl 0.5 gram per cent, K_2HPO_4 0.5 gram per cent.

Glucose agar: Water 1000 cc. agar 15.0 grams, glucose 10.0 grams, K_2HPO_4 0.5 gram, NH_4Cl , KNO_3 , asparagin or peptone 0.5 gram per cent.

Czapek's agar: Distilled water 1000 cc., K_2HPO_4 1.0 gram, MgSO_4 0.5 gram, KCl 0.5 gram, FeSO_4 0.01 gram, NaNO_3 2.0 gram, sucrose 30.0 gram, agar 15.0 gram.

The substitution of glucose or glycerin for sucrose makes this medium more favorable for the growth of these organisms.

Malate-Glycerin agar: Calcium Malate agar + 1 per cent glycerin.

medium, but on other environmental conditions and on the nature of the mother culture. When many strains are accumulated we find that one organism will approach another organism and often a culture is obtained which can hardly be differentiated from either of the two. The differences in physiological activities are often not qualitative but quantitative. The dominant idea in the following investigations has been not merely the accumulation of more data for the differentiation of the species, but the study of the metabolism of these species so as to find their proper place in nature, both as individuals and as groups consisting of allied species.

The uses of the terms *Actinomyces*, *Streptothrix* and *Nocardia*, have been reviewed by Waksman and Curtis (1916), Krainsky (1914) and Conn (1917); the term *Actinomyces* will be used for reasons given in the above mentioned papers.

A preliminary description of the organisms isolated from the soil by Waksman and Curtis has appeared elsewhere (1916) and a full description will be published at a later date.

The following organisms were used in these investigations:

A. violaceus-ruber W. & C.

A. violaceus-acesari W. & C.

A. scabies (Thaxter) Güssow (Syn. *Oospora scabies* Thaxter, *A. chromogenus* Gasperini?) isolated from potato scab.

A. exfoliatus W. & C.

A. diastaticus (Krainsky?) W. & C.

A. albus (Krainsky?) W. & C.

A. reticuli W. & C.

A. citreus (Krainsky?) W. & C.

A. griseus (Krainsky?) W. & C.

A. verne W. & C.

A. alboflavus W. & C.

A. albosporeus (Krainsky?) W. & C.

A. bobili W. & C.

A. californicus W. & C.

A. lipmanii W. & C.

A. rutgersensis W. & C.

A. aureus W. & C.

A. halstedii W. & C.

A. fradii W. & C.

A. roseus (Krainsky?) W. & C.

A. lavendulae W. & C.

A. poolensis Taubenhaus.

A. pheochromogenus Conn.

96, 120, 128, 145, 154, 161, 168, 190, 198, 202, 205, 206, 214, 215, cultures isolated from different soils by Waksman and Curtis.

For the following four cultures the writer is indebted to Dr. K. F. Meyer of the Hooper Foundation, San Francisco.

A. madurae: Received from A. M. N. H. by Parke, Davis Company (no. 05), received by them in May 1902, from Král.

A. hominis: isolated by Foulerton from abscess of palm of hand in 1911.

A. bovis: Received from A. M. N. H. no. 49, received by them from Parke, Davis and Company (no. 04).

A. asteroides (*A. eppingeri*): Received from Pasteur Institute, 1914.

II. THE ACTION OF ACTINOMYCETES UPON MILK

Historical

The importance of milk as a culture medium for the study of bacteria has long been recognized by workers in bacteriology; although milk is a very complex medium, it is more or less standard in composition and the reactions produced upon it by microorganisms are so characteristic, that it has found general acceptance.

The growth of an organism upon milk should be studied from several angles: the action upon the proteins and sugars of the milk, and the production of different enzymes must all be considered. Nitrogen is present in fresh skim milk as casein, about 3 per cent, albumin, about 0.5 per cent, and other nitrogenous compounds, about 0.05 per cent. The casein is in the form of calcium caseinate, which is acid to phenolphthalein and neutral to litmus. The calcium present in the milk is just sufficient to keep the casein in solution; when a large excess of

calcium salt is added to the milk, the casein is precipitated; upon addition of acid to milk, the calcium is converted into the corresponding salt and the free casein is precipitated, producing the well known phenomenon of coagulation. The coagulation of the milk may be due to two different causes: it may result from the action of an acid upon the milk, as just pointed out, or the curdling may be due to enzyme action, as in the case of rennin (chymosin, lab), which transforms casein into paracasein and produces coagulation, in the presence of calcium salts.

Milk contains as high as 6 per cent of sugar, mostly lactose, but with small quantities of glucose also. A permanent acid reaction of the milk is induced by the organisms that ferment lactose, but if an organism can only utilize glucose the reaction is usually at first acid, later changing to alkaline due to protein decomposition or the production of carbonates. The accumulation of acid due to the fermentation of lactose may be sufficient to cause an acid coagulation of the casein, and this is very often the type of coagulation which milk undergoes as a result of the action of bacteria. Savage (1904) demonstrated that curdling of milk by *B. coli* is due, in almost all cases, to the formation of acid, presumably lactic acid by the bacteria. Curdling of the milk produced by the action of an enzyme can be easily distinguished from that produced by an acid: when the acid is neutralized, the latter type of curd will redissolve, but not the former: O'Heir (1906) has shown that an acid coagulum in milk is insoluble in weaker alkalies, but dissolves readily in stronger ones such as NaOH. Savage (1904) demonstrated further that rennin will not coagulate boiled milk, or only with great difficulty, this being due to the precipitation of soluble salts of calcium in the boiling. When freshly separated and pasteurized milk is used, it is possible that the bacteria left in the milk may ferment the lactose and that the lactic acid thus produced may affect favorably the action of rennin.

Besides these primary reactions induced by the growth of microorganisms in milk, secondary reactions have to be taken into consideration also: such as the peptonization of the coagulum. This is due to the proteolytic activities of the organism, which

splits the paracasein by means of an enzyme into proteoses, peptones, polypeptides, amino acids, and ammonia. The milk may also clear up, without any previous coagulation. Van Slyke and Bosworth (1916) have shown that in the souring of milk an increase of the nitrogen of the whey takes place, and that this increase is due to the albumin. Kendall and his associates (1914) studied the growth of a number of bacteria on milk; many different transformations were produced in the milk as a result of the metabolism of particular organisms. *B. coli* produced a marked lactic acid fermentation with acid coagulation of the casein; *B. proteus* did not ferment lactose but attacked the proteins and at the end of the third day the milk was coagulated and later peptonized; *B. subtilis* and *B. mesentericus* acted in a similar manner, the first producing an alkaline and the second an acid reaction, and so on. These different reactions upon the milk can be easily explained by the differences in the metabolism of these organisms.

It is assumed that the enzyme produced by the microorganisms that clot milk is of a nature similar to the animal rennin, lab, or chymosin. Duclaux (1907) claimed that bacteria act upon milk in three different ways: first, by producing a rennet-like enzyme which coagulates the milk, without making it more available for their nutrition; secondly, by producing a casease, an enzyme which digests the clot; this enzyme acts also upon the albuminoids of the milk but more actively upon the casein; thirdly, the microorganisms may produce a decomposition of the milk by a process of nutrition; if the casease is in excess over the rennet, the milk in this case will be decolorized without any previous coagulation.

Babcock and associates (1899), differentiated between the enzymes of animal origin and those produced by bacteria. He stated that the products formed by the galactase normally present in the milk resemble those produced by the liquefying or peptonizing bacteria rather than those formed by the enzymes of animal origin; trypsin, pancreatin and pepsin produce no ammonia, while galactase and the proteolytic enzymes from bacterial cultures do produce ammonia.

The chemistry of the rennet action is still a disputed point. When coagulation takes place, the bulk of the casein is precipitated as paracasein, which is very similar to casein itself; whether the decomposition of casein occurs is still uncertain. Some investigators, such as Pawlow and Parastschuk (1904), Sawjalow (1905), Sawitch (1908), Gewin (1907), and others, believe that the action of pepsin and rennet result from the same enzyme, the clotting of milk being regarded as the commencement of peptic digestion; others, such as Nencki and Sieber (1901), Pekelharing (1902), Taylor (1909), and particularly Hammarsten (1908) are of the opinion that the two enzymes are not identical. Hammarsten (1915) further states that both enzymes act proteolytically, but under different conditions, rennet acting at a lower acid concentration than pepsin, and also that the optimum temperature for the two enzymes is different. The casein molecule is split up into a larger molecule (Käse) and a smaller (Molkeeiweiss). The large molecule is rendered insoluble by the presence of soluble calcium salts and forms the clot. Schryver (1913) and Mellanby (1913) consider the rennet clot as probably a combination of enzyme and protein. Bosworth (1913) and Van Slyke and Bosworth (1916) believe that the ferment breaks up the casein molecule into two molecules of paracasein, each half the size of the original molecule; and that small quantities of CaCl_2 render the paracasein insoluble. Rennin action is probably a hydrolytic cleavage and may be considered the first step in the proteolysis of casein; the action attributed to rennin may be produced by any proteolytic enzyme.

Without going into further detail in regard to the chemical transformations of the milk constituents and the rôle of microorganisms in these transformations, a few words should be said here concerning the use of milk for the study of the Actinomycetes by the earlier investigators. Lehmann and Neumann (1912) reported that *A. bovis* Harz was found to produce no change in milk in eight days; *A. farcinicus* Gasperini dissolved the casein without coagulation, the reaction becoming alkaline; *A. chromogenus* Gasperini clarified the milk giving an alkaline reaction. Petruschky (1912) reported that *A. madurae* Vincent

coagulated the milk and later redissolved it, while Besson (1912) stated that *A. madurae* grew in milk, but that no coagulation took place. Goadby (1903) found that *A. buccalis* isolated from the mouth produced no change for the first two days in milk at 37.5°C.; later the milk was precipitated and then cleared, the reaction becoming slightly alkaline. Lutman and Cunningham (1914) found that *A. chromogenus* (*A. scabies*) digested the milk slowly without coagulum, the milk becoming alkaline. These few observations do not throw much light upon the action of the Actinomycetes upon milk, the only point that seems definite being the tendency for the reaction of the milk to become more alkaline as a result of the action of these organisms. All the Actinomycetes, according to Krainsky (1914), attack casein readily and split it by means of an enzyme, with the production of ammonia. Nadson (1903) stated that the Actinomycetes isolated from the curative mud of the Veisov salt lake produce a precipitate of the casein of the milk. The clot is then dissolved gradually from the surface downward and the milk is transformed into an almost transparent fluid, having a reddish opalescence; the liquid is distinctly alkaline and a small portion of undissolved casein is left at the bottom of the tube.

A preliminary paper was recently published by the writer (1918) on the metabolism of pathogenic Actinomycetes, in which the question of the action of these organisms upon milk was discussed, but since it was brief and preliminary the results will be included in table 1 for comparative purposes.

Experimental

The organisms used in this work were isolated from the soil in the end of 1915 by Waksman and Curtis and briefly described in 1916. *A. madurae*, *A. hominis*, *A. bovis*, *A. asteroides* and *A. scabies* were obtained from sources noted above. A full description of the organisms indicated by numbers as well as of those previously described will appear later. All the organisms were grown upon a synthetic medium,¹ with the exception of the four animal pathogens.

Freshly separated milk was placed in tubes and flasks and sterilized for thirty minutes on three consecutive days in flowing steam at 100°C. The sterile milk was then inoculated from the different cultures grown on the synthetic medium and incubated at 37°C. At the end of the incubation period amino nitrogen and ammonia were determined in some of the cultures, the amino nitrogen by the use of the micro-apparatus of Van Slyke (1914); the ammonia by the Folin aeration method, as modified by Steele (1910).

The indicator suggested by Clark and Lubs (1917), dibrom-orthocresolsulfonphthalein, or more simply "bromocresol purple," was found to be excellently suited for the study of reaction changes in milk and was used in place of litmus for this purpose.

The indicator was dissolved in NaOH solution and added to the fresh, unadjusted milk in the proportions suggested by Clark and Lubs (1917). Since it is very difficult to describe the color changes of the indicator in solution and we have no standards for determining colorimetrically the P_H values of milk satisfactorily, the changes in reaction produced as a result of the growth of the Actinomycetes on milk are indicated as follows: 0 designates no change in reaction (about P_H 6.3); — acid; + weakly alkaline; ++, +++, +++++ designate different degrees of alkalinity, the last being the most alkaline cultures as indicated by the use of the particular indicator.

The results brought out in table 1 point definitely to the fact that the chemical changes produced in milk due to the action of Actinomycetes can be taken advantage of in the identification of the species of these organisms. The chemical changes of the milk constituents seem to be quite definite for different species of Actinomycetes. The results brought out in the above table were duplicated several times and where discrepancies occurred attention will be called to them in the following discussion.

First we find several organisms, such as *A. griseus*, *A. poolensis*, *A. madurae*, 206, 214, 215, which coagulate the milk in three to five days, soon begin to peptonize the curd, and have digested it all in about ten days. The amount of amino nitrogen and ammonia produced by these organisms is very great; for example,

TABLE 1
The action of *Actinomyces* upon milk*

NAME OF ORGANISM	COAGULATION	PEPTONIZATION			HYDROLYSIS	NO ACTION	BROWN PIGMENT	NH ₄ -N IN 15 DAYS		NH ₄ -N IN 40 DAYS		REACTIONS†
		Start	End	Rapidity‡				Milli-grams in 10 cc.	Per cent	Milli-grams in 10 cc.	Per cent	
	days	days	days	days								
Control (unincubated).	—	—	—	—	—	—	—	1.33	2.2	1.33	2.2	Unad-justed
Control (incubated).....	—	—	—	—	—	—	—	5.11	8.6	2.65	4.4	0
<i>A. aureus</i>	—	—	—	—	—	—	—	34.40	57.7	14.82	24.9	0
<i>A. griseus</i>	4-5	5	10	+++	—	—	—	4.56	7.6	33.21	54.0	+++
<i>A. californicus</i>	15	15	Not in 45	+	—	—	—	6.80	11.5	17.96	30.1	+
<i>A. viridochromogenus</i> †...	5-6	6	12	+++	—	—	—	9.12	15.4	23.66	39.7	+
<i>A. albus</i>	—	—	—	—	20	—	—	8.76	14.7	25.94	43.5	+++
<i>A. diastalticus</i>	5-7	7	18-25	++	—	—	—	5.69	9.5	19.95	33.5	+
<i>A. fradii</i>	10-12	12	20	++	—	—	—	—	—	21.38	35.9	+++
<i>A. violaceus-ruber</i>	—	—	—	—	12-15	—	—	—	—	20.80	34.9	+
<i>A. violaceus-caesari</i>	10-12	12	50	+	—	—	—	13.45	23.4	15.96	26.8	+
<i>A. purpeo-chromogenus</i> ...	10	10	Not in 50	+	—	—	—	14.25	23.9	14.25	23.9	+++
<i>A. exfoliatus</i>	—	—	—	—	8-10	—	—	7.10	11.9	16.18	27.1	0
<i>A. reticuli</i>	4-6	6	Not 50	+	—	—	—	—	—	25.94	43.5	+
<i>A. 180</i>	4-6	6	Not 50	+	—	—	—	—	—	26.32	44.2	+++
<i>A. citreus</i> †.....	9-10	10	20	+++	—	—	—	—	—	28.50	47.8	+++
<i>A. alboflavus</i>	—	—	—	—	10-12	—	—	—	—	9.41	15.8	+
<i>A. verne</i>	4-5	5	18	+++	—	—	—	—	—	26.79	44.9	0
<i>A. albosporus</i>	—	—	—	—	—	—	—	—	—	23.37	39.2	+++
<i>A. bobili</i>	—	—	—	—	15-18	—	—	5.8	9.7	22.80	38.3	+++
<i>A. lipmanii</i>	8-9	9	30	++	—	—	—	4.30	7.2	11.69	19.6	+++
<i>A. vulgarensis</i>	5-6	6	Not 50	+	—	—	—	—	—	—	—	+++
<i>A. roseus</i>	—	—	—	—	10-15	—	—	—	—	—	—	+++

	—	—	—	20-30	+	35.63	15.68	26.3	5.8	9.7	++
<i>A. lavendulae</i>	—	3-4	10-20	—	—	59.8	—	—	—	—	++
<i>A. madurae</i>	3-4	3-4	10-20	—	—	19.95	—	—	—	—	++
<i>A. hominis</i>	5-6	5-6	20	—	—	33.5	—	—	—	—	++
<i>A. bovis</i> †.....	10-12	12	40	—	—	16.8	—	—	—	—	+
<i>A. aseleroides</i>	—	—	—	—	—	5.60	9.6	—	—	—	0
<i>A. halstedii</i>	10	10	Not 50	—	—	—	—	—	—	—	++
<i>A. pooleensis</i> †.....	4-5	4-5	10	—	—	20.60	34.6	11.69	19.9	3.2	++
<i>A. scabies</i> I.....	5-10	5-10	10-30	—	—	12.83	21.5	30.21	50.7	21.8	++
<i>A. scabies</i> II.....	10-12	12	15-30	—	—	9.22	15.5	12.83	21.5	15.3	++
<i>A. phoechromogenus</i>	—	—	—	—	—	4.85	8.1	5.70	9.6	3.7	+
	(soft clot often)										
214.....	3-4	3-4	8-10	—	—	31.64	53.1	31.64	53.1	14.4	++
215.....	3-4	3-4	7-10	—	—	21.95	36.8	21.95	36.8	12.0	++
96.....	10-20	10-20	30	—	—	21.95	36.8	21.95	36.8	11.8	++
128.....	3-6	3-6	15-30	—	—	7.89	13.2	25.65	43.0	12.7	+
145.....	6-7	7	Not 50	—	—	5.40	9.0	5.70	9.6	3.5	0
154.....	4-5	5-6	Not 50	—	—	23.66	39.7	23.66	39.7	6.7	0
161.....	6-7	7-8	Not 50	—	—	21.66	36.3	21.66	36.3	10.8	++
168†.....	5-8	6-8	20	—	—	16.96	28.5	16.96	28.5	13.2	+
190.....	4-6	5-6	15-30	—	—	31.92	54.2	31.92	54.2	23.3	++
198†.....	5-6	5-6	18	—	—	16.53	27.7	16.53	27.7	4.8	++
202.....	—	—	—	—	—	10.55	17.7	10.55	17.7	5.3	0
205.....	—	—	—	—	—	13.12	22.0	13.12	22.0	4.3	++
206.....	4-5	4-5	10-12	—	—	28.32	47.5	28.32	47.5	13.7	++
<i>B. subtilis</i>	2	2	6-8	—	—	18.81	31.6	18.81	31.6	15.4	++
<i>B. mycoides</i>	2	2	6-8	—	—	21.09	35.4	21.09	35.4	19.0	++
<i>B. coli</i>	6	0	—	—	—	3.14	5.3	3.14	5.3	1.7	acid

*Total nitrogen in 10 cc. of milk is 59.6 mgm.

Hydrolyzed also in one or more cases.

— Negative, + weak, ++ fair, +++ rapid, ++++ very rapid.

§ 0 designates no change in reaction, +, ++, +++ designate different degrees of alkalinity, ++++ being the most alkaline reaction.

A. griseus converted in fifteen days 57.7 per cent, *A. madurae* 59.8 per cent and *A. poolensis* 34.6 per cent of the nitrogen of the milk into amino-nitrogen. *A. griseus* converted 23.3, *A. poolensis* 21.8, 214–24.1, 215–20.1, 206–23.0 per cent of the nitrogen of the milk into ammonia in forty days.

The reaction of the milk becomes highly alkaline in this series of organisms; this alkalinity is no doubt due to the production of large quantities of ammonia and other basic substances and possibly to the production of carbonates from the milk sugars.

The second group of organisms contains those species that coagulate the milk rapidly, but peptonize the curd slowly, so that in most cases the curd is not digested even in forty-five days. In this group of organisms we would include *A. reticuli*, 43 and 120, *A. rutgersensis*, 145, 154, 161. If the rennin and the proteolytic enzymes are distinct, it would seem that these organisms produce as strong a rennet-like enzyme as the first group, but a weaker proteolytic enzyme, which therefore accounts for the slow peptonization of the curd. This group of organisms is further characterized, in general, by the production of a low alkalinity, particularly in the case of the *A. reticuli* cultures and 145 which produced almost no change in the reaction of the milk. This fact is in accord with the assumption that the production of a proteolytic enzyme by these organisms is limited.

Several organisms, namely *A. hominis*, 128, *A. viridochromogenus*, *A. diastaticus*, *A. verne*, stand between the two groups, producing a rapid coagulation, the peptonization being more slow than in group I and more rapid than in group II. Since this division has no sharp lines of demarcation and is purely artificial, the intermediate organisms can be considered as related to both of these groups.

Those organisms which coagulate the milk only in ten to twelve days followed by a rather rapid peptonization would form a third group. Here we would include such organisms as *A. fradii*, *A. lipmanii*, *A. bovis*, *A. scabies*, 96, *A. citreus* and 168. These organisms are characterized by an alkalinity lower than group I but higher than group II, no doubt due to the fairly rapid digestion of the clot.

A fourth group would comprise those organisms which do not coagulate the milk, but hydrolize it without previous coagulation. Here we would include *A. albus*, *A. exfoliatus*, *A. violaceus-ruber*, *A. bobili*, *A. lavendulae*, *A. roseus* and *A. alboflavus*. These are all characterized by a very high alkalinity. It is possible that the lack of coagulation is not due to the lack of a rennet-like enzyme, but merely to the fact that the digestion of the casein of the milk proceeds rapidly at its early stages, so that coagulation is not observed or is absent. That some of the organisms that coagulate the milk may produce hydrolysis without clotting can also be concluded from the fact that a few cultures, namely, *A. fradii*, *A. bovis*, *A. scabies*, *A. citreus* and 198 produced hydrolysis of the milk in some cases, while in other cases the same organisms produced a clot and then digested it. This will explain the fact that *A. bovis* and *A. scabies* (*A. chromogenus*) are able, as reported by some investigators, to coagulate the milk and then peptonize it, while others state that these organisms can only hydrolize the milk without previously coagulating it. It is interesting to note that all these organisms which hydrolized the milk without previously clotting it at one time or produced a clot and then digested it at another time, were placed in group III, which contains the organisms that produce a clot late but peptonize the clot rapidly. The possible explanations would be either that these organisms produce rennin at one time and not at another, which is hardly probable, or that the clot formation and the peptonization of the casein go hand in hand so that coagulation is often not observed. The interaction between casein and the proteolytic enzyme may be more rapid than between casein and rennin. The lack of clot may therefore not necessarily indicate the lack of production of a rennet-like enzyme, as will be pointed out again in another connection.

The fifth group consisted of strains which seemed to have no effect upon the milk in thirty days. Some of these organisms are probably unable to grow in milk and could not be reisolated; *A. aureus*, *A. pheochromogenus*, 202, 205 belong to this group. In the case of the organisms that did grow upon the milk, such as *A. asteroides*, the action upon the casein is so slight as to

produce no visible change. These organisms seem to produce a very weak proteolytic enzyme and no rennet action; a longer incubation period (forty-five days) will indicate a degree of fair digestion of the milk protein.

The species belonging to this group are mostly chromogenus types, i.e., producing black pigments on organic media. When grown on milk at 25°C., all of them produced a surface brown to black ring, accompanied by an imperfect clot formation and weak digestion; the amino nitrogen content of the milk was rather low and the reaction quite alkaline (++ or +++).

In general when grown at 25°, nearly all the species produced a better growth upon the milk, but the action upon the milk, ran about alike to that obtained at 37°.

The Actinomycetes are variable in nature and the amount of growth upon different media depends upon many factors, such as mother cultures, temperature of incubation, etc. We would therefore expect that, at different temperatures of incubation, some species at least, would show distinctive differences in the clot formation, rapidity of peptinization and change of reaction. These differences were actually obtained but they varied in degree rather than in kind.

The division of the Actinomycetes into five groups according to their action upon the milk constituents should be looked upon as only an attempt to point out in a definite manner a possible differentiation of these organisms. Although, upon repeated transfer, the same reactions in milk were generally obtained, there were several exceptions and therefore the division lines cannot be absolute. The production by some organisms of a clot at one time and hydrolysis of the milk, without previous clotting, at another has already been pointed out.

A. madurae produced a clot in three to four days and then digested it rapidly; but, when grown for a long time upon synthetic media, it clotted the milk in about the same period of time but digested it slowly. This may perhaps be due to the nature of the medium upon which the organism has been grown previously. Such an effect of acclimatization upon the biochemical activities of microorganisms has not been studied in detail as

yet, although we know that it is important. For example, it is known that the production of enzymes by microorganisms depends upon the nature of the medium upon which they are grown, but we know also that this difference is of a quantitative rather than a qualitative nature. Hardly any attention has ever been paid to the influence of the medium on which an organism was grown, upon the production of enzymes by this organism, when transferred into a new medium. When more information on this subject has been accumulated, some of the discrepancies observed in the growth of Actinomycetes on milk, which are doubtless enzymatic phenomena, will probably be explained.

The change of the reaction of the milk is very characteristic. In nearly all cases the milk became alkaline; in only a few instances did the reaction fail to change; in no instance did the reaction become acid. The amount of alkalinity seems to go hand in hand with the amount of digestion. When the coagulum was produced there was usually no change or very little change of reaction; when the digestion began, the reaction became more alkaline and continued to increase with the advance of the digestion, proceeding from surface to bottom.

When the two periods of incubation are compared, it becomes evident that greater differentiation can be observed for the different cultures, if a record is taken at the end of a shorter period of incubation, namely about fifteen days at 37°. Since most of the organisms are active proteolytically and are able to digest the milk proteins, a long incubation period will tend towards the elimination of the differences, as observed by inspection of the cultures. For example, *A. griseus*, one of the most active Actinomycetes studied, almost completely digested the milk proteins in fifteen days, producing 57.7 per cent amino nitrogen and about 20 per cent ammonia nitrogen, while at forty-five days these quantities were changed to 54.0 and 23.3 per cent. This would indicate that very little splitting of proteins took place between the two incubation periods, and that some amino nitrogen compounds were reduced to ammonia. On the other hand the cultures of weaker proteolytic organisms, such as *A. diastaticus* and *A. fradii*, which contained at the end of fifteen days only 14.7

and 9.5 per cent amino nitrogen, have shown in forty-five days an increase to 43.5 and 33.5 per cent respectively.

The comparatively large quantities of amino nitrogen and ammonia produced by some Actinomycetes from milk proteins would indicate that some of these organisms may be very important in the splitting of proteins in nature. Their activity becomes more evident when compared with the action of several common bacteria. *B. mycoides* and *B. subtilis*, which are among the most active proteolytic bacteria known, produced from the milk proteins, with the same period and temperature of incubation, less amino nitrogen and only slightly larger quantities of ammonia nitrogen than the Actinomycetes placed in group I; *B. coli*, which is known as a weak proteolytic organism, produced an acid clot, and split the milk proteins only to a very limited extent.

To obtain further information in regard to the action of the Actinomycetes upon the milk and to determine whether the phenomena observed were due to enzyme activities or to the growth of the cells, the following experiments were carried out:

One cubic centimeter of the fifteen-day milk culture of the organisms (in case the coagulum was partly or fully digested, the whey was used) was added to 10 cc. of fresh sterile milk in test tubes, using about 1 cc. of toluene to prevent any growth from taking place.

To test also the proteolytic action of the culture, 1 cc. of the fluid was added to 10 cc. of sterile gelatin, 15 per cent in distilled water, using 1 cc. of toluene for each tube. The tubes were then stoppered with rubber stoppers and incubated at 37°C. For examination, the gelatin cultures were placed for one hour on ice, then examined; if they remained liquid, they were pronounced digested and if they solidified again, undigested.

The results are given in table 2.

The production of a rennet-like and proteolytic enzyme is definite, although we might argue from the data presented in table 2 that both activities can be attributed to the same enzyme, and that the difference between the proteolytic and rennet-like action depends entirely upon the velocity of the action of the enzyme.

For example, *A. griseus*, which seems to possess a strong proteolytic enzyme, and which always clotted the milk very rapidly, did not produce any clot when only the enzyme was studied, but rapidly peptonized (hydrolized) the milk and liquefied the gelatin. So much of the proteolytic enzyme had been added that the digestion of the casein of the milk proceeded very rapidly and no clotting was observed. In the case of the other organisms which possess less active proteolytic enzymes, as indicated by

TABLE 2
The production of rennet-like and proteolytic enzymes by Actinomycetes

NAME OF ORGANISM	COAGULATION OF MILK	PEPTONIZATION OF COAGULUM	HYDROLYSIS	LIQUEFACTION OF GELATIN
	days	days	days	days
<i>A. griseus</i>	—	—	2	1
<i>A. griseus</i>	1	4	—	1
<i>A. diastaticus</i>	3	10	—	3
<i>A. lipmanii</i>	4	Slow	—	5
<i>A. californicus</i>	4	Slow	—	5
<i>A. albus</i>	—	—	—	5
<i>A. viridochromogenus</i>	4	Slow	—	2
<i>A. poolensis</i>	3	8-10	—	Not determined
<i>A. citreus</i>	2	7-10	—	Not determined
96.....	—	—	6	Not determined
<i>A. scabies</i>	—	—	—	—
<i>A. pheochromogenus</i>	—	—	—	—
<i>A. aureus</i>	—	—	—	—
<i>A. reticuli</i>	—	—	—	—
<i>A. exfoliatus</i>	—	—	15	Not determined
198.....	12	30	—	Not determined
215.....	4	8-10	—	3

the period of time necessary for the liquefaction of the gelatin and the peptonization of the milk, the milk was clotted. Organism 96, which clotted the milk very slowly (see table 1), did not show any rennet-like enzyme although peptonization of the milk (hydrolysis) took place. This may add weight to the assumption that the rennet-like and proteolytic enzymes are one and the same thing, the rennet-like action being merely the first step in the action of the proteolytic enzyme, but when the enzyme is

either too active, as in the case of *A. griseus*, or too inactive, as in the case of 96, no clotting of the milk will be observed. The organisms that produced no visible action upon the milk, or acted upon it very slowly, did not exhibit any active proteolytic enzymes.

To obtain a more or less pure enzyme for work, *A. griseus*, *A. exfoliatus* and *A. lipmanii* were grown in 100 cc. quantities of sterile milk in 200 cc. Erlenmeyer flasks for twenty days. At the end of that period, *A. griseus* and *A. exfoliatus* cultures were wholly and *A. lipmanii* cultures largely peptonized. The clear filtered fluid was precipitated by means of 95 per cent alcohol; the precipitate was washed with absolute alcohol and ether, and dried over sulfuric acid. The dried material was added (about 10 mgm.) to 10 cc. of sterile milk and incubated at 37°; toluene was used to prevent contamination. The enzymes of the three organisms produced a complete clot of the milk in twenty-four hours. The clot was not digested readily even after ten to twelve days incubation at 37°. A proteolytic and rennet-like enzyme is therefore shown to be certainly present in some species of Actinomycetes.

The last experiment would lead to the assumption that the rennet-like enzyme can thus be shown to be distinctly different from the proteolytic enzyme. *A. griseus* and *A. exfoliatus* were grown again in 300 cc. quantities of sterile milk in 1 liter Erlenmeyer flasks till all the milk was digested (*A. griseus* first produced a clot in six to seven days with perfect digestion in twelve to fifteen days; *A. exfoliatus* hydrolized the milk in fifteen days). The milk was filtered through paper and the filtrate precipitated by means of alcohol, redissolved in water and reprecipitated; then washed with absolute alcohol and ether and dried over sulfuric acid. The material was scraped off the paper and added to a series of tubes of milk (about 5 to 10 mgm. to 10 cc. of milk). A perfect clot was produced in all cases without any whey formation for the *A. exfoliatus* and only a small quantity (0.5 to 0.75 cc.) for the *A. griseus* in ten to fifteen days at 37°. The whey of the latter contained 4.03 mgm. amino nitrogen per 10 cc. of whey, indicating a very faint digestion which is no doubt

due to a trace of the proteolytic enzyme precipitating with the rennet-like enzyme. It is interesting to note that *A. exfoliatus* which, when grown on milk, produced no clot but merely hydrolyzed the milk gave a strong rennet-like enzyme.

Several Actinomycetes were grown in Czapek's synthetic solution for thirty days in Erlenmeyer flasks at 22°. At the end of that period the cultures were filtered and both filtrate and mycelium used for study of the presence of rennet-like and proteolytic enzymes, the former containing the exoenzymes and the latter the endoenzymes. The mycelium was washed in water several times, then treated by the "acetone-dauerhefe" method (ten minutes in acetone, three minutes in ether and dried over sulfuric acid).

One cubic centimeter of the filtrate and 25 mgm. of the treated mycelium were added to test tubes containing 10 cc. of sterile milk, using toluene to prevent contamination. The tubes were incubated at 37°. The results are given in table 3.

The data presented in table 3 point to the fact that both types of active proteolytic enzymes are produced by Actinomycetes when grown on synthetic media. Several interesting observations can be made from these data. The exoenzyme (filtrate) has very active rennetic properties, as shown by the formation of a clot, and rather weak proteolytic properties, as shown by the fact that either the coagulum was not digested, as in the case of *A. aureus* and 168, or was digested only slowly (*A. fradii*) or to a very limited extent. The endoenzyme (mycelium) contained a weak rennet-like enzyme or none at all, but a very active proteolytic enzyme, as shown by the amino nitrogen in the medium. It is interesting to note that *A. aureus*, which did not produce any rennet-like endoenzyme, does not clot the milk when grown upon that medium, and 168, the ectoenzyme of which did not clot the milk, but hydrolyzed it, often produces a hydrolysis of the milk when grown upon that medium.

It would look as if the rennet-like enzyme is dissolved out into the medium, while the proteolytic enzyme is kept largely within the mycelium, this tending to indicate a distinct difference between the two enzymes.

TABLE 3

The production of rennet-like and proteolytic enzymes by Actinomycetes grown upon synthetic media

NAME OF ORGANISM	EXOENZYME				ENDOENZYME		
	Number of cubic centimeters	Coagulation days	Digestion days	NH ₄ -N in 10 cc.	Coagulation days	Digestion days	NH ₄ -N in 10 cc.
<i>A. diastaticus</i>	1	4	Trace	—	4	$\frac{1}{2}$ in 15	9.7
	2	4	Trace	—			
	3	2	$\frac{1}{2}$ in 15	7.3			
<i>A. aureus</i>	1	15	Trace	—	0	0	5.9
	2	12	Trace	—			
	3	4	Trace	—			
<i>A. fradii</i>	1	2	Complete in 15-30	11.7	3	10	25.0
	2	2	Complete in 15-30	—			
	3	1	Complete in 15-30	—			
168.....	1	4	Trace	—	0	Hydrolysis	24.5
	2	2	Trace	—			
	3	2	Trace	—			

SUMMARY

1. The Actinomycetes vary greatly in their action upon milk.

2. These organisms can be divided into five groups, using as a basis their action upon milk, although no sharp lines can be drawn between the different groups which blend into one another: group I contains those organisms that coagulate the milk rapidly and then peptonize the coagulum rapidly; group II, organisms that coagulate the milk rapidly, but peptonize the coagulum slowly; group III, those organisms which coagulate the milk slowly, but then peptonize the coagulum fairly rapidly; the organisms that peptonize (hydrolize) the milk, without coagulating it, will form group IV; and group V includes those organisms that have no visible action upon the milk. The differences will

be observed more readily when the period of incubation at 37°C. is not too long.

3. A few species do not lend themselves readily to this system of classification and are to be grouped in positions intermediate between two of these groups.

4. A few species give variable results on repeated inoculation in milk; this variability can be explained when the metabolism of the proper organisms is taken into consideration.

5. Rennet-like and active proteolytic enzymes are produced by the organisms that exert a strong proteolytic action upon the milk.

6. The reaction of the milk is changed in nearly all cases to an alkaline one.

7. The rennet-like and proteolytic enzymes, produced by the Actinomycetes, seem to be distinct from one another.

III. THE USE OF BLOOD MEDIA FOR THE STUDY OF ACTINOMYCETES²

The Actinomycetes were studied on blood media for two reasons: firstly, to find out whether these media, which are well adapted for the growth of most pathogenic bacteria, can also be used successfully for the study of Actinomycetes; secondly, to see whether growth on these media brings about characteristic reactions, which indicate the metabolism of these organisms and help in their proper identification.

Schlegel (1913) states that Israel and Kischevsky found coagulated serum to be a good medium for the growth of Actinomycetes. *A. bovis* was reported by some investigators to grow well on blood serum with the liquefaction of the serum, and *A. madurae* was reported not to produce any growth on blood serum. Goadby (1903) stated that *Streptothrix buccalis* isolated from the mouth grew on blood serum (at 37.5°C.) and liquefaction of the serum was noted. Macé (1905) stated that *Cladothrix* (*Actinomyces*) develops well in liquid blood serum, producing a browning of the medium and a characteristic odor. After several months, the

² The work included in this paper was partly done by the writer at the Cutter Biological Laboratories, Berkeley, Cal.

liquid becomes more fluid and, while it does not coagulate upon heating, gives a slight floccose precipitate on boiling. The liquid contains ammonia, pro-peptones, crystals of tyrosin, leucine and glycocoll, but no indol. This species was isolated by Macé from the soil and was found to be one of the active agents in the transformation of albuminous matter. The writer (1918) has recently pointed out that some pathogenic Actinomycetes can liquefy blood serum and produce hemolysis of whole blood medium, while others do not; the Actinomycetes seem to vary markedly in this respect.

These observations would seem to point out the fact that at least some Actinomycetes are active proteolytically when blood proteins are present as a substratum.

Both whole blood and blood serum were used for this work. The blood agar was prepared as follows: nutrient agar containing veal infusion (500 grams of veal per liter of tap water, boiled ten minutes and filtered), 1 per cent Bacto-peptone, 0.5 per cent sodium chloride, 2.5 per cent agar, adjusted to $+ 1.0$ ($P_H = 7.6-7.8$) flaked and sterilized. After sterilization, the agar was cooled to 45 to 50°C. and about 10 per cent of sterile rabbit blood was added, the flask was well shaken, and the agar poured, under sterile conditions, into sterile test-tubes or sterile Petri dishes; the tubes were slanted, and both tubes and dishes incubated for forty-eight hours to insure sterility.

The Loeffler's blood serum was prepared according to the usual formula: to 3 parts of ox-blood serum 1 part of glucose bouillon was added containing veal or beef infusion prepared as before, 1 per cent each of peptone and glucose and 0.5 per cent sodium chloride, reaction $+ 1.0$ ($P_H 7.6$ to 7.8); these were well mixed, passed through a Berkefeld filter, tubed into sterile tubes, using sterile containers, slanted in the Arnold, coagulated and sterilized, the process lasting for two hours, the temperature never going up above 90°C. The tubes were incubated for forty-eight hours to insure sterility.

Growth of Actinomycetes on blood agar

The organisms grown upon the synthetic medium (Czapek's) were inoculated upon the blood agar, tubes and plates, and cultures incubated at 37°C. In nearly all instances a good growth was obtained in twenty-four to forty-eight hours, with the exception of *A. pheochromogenus* which grew very slowly; blood agar forms, therefore, a good medium for the growth of the Actinomycetes.

TABLE 4

The growth of Actinomycetes upon blood agar and the production of hemolysis

NAME OF ORGANISM	COLOR OF COLONY	SOLUBLE PIGMENT	AERIAL MYCELIUM	HEMOLY- SIS
<i>A. aureus</i>	Dark brown	Dark	—	0
<i>A. albus</i>	Green	—	White	0
<i>A. griseus</i>	Greenish	—	White	+++
<i>A. diastaticus</i>	Grayish brown	—	—	++
<i>A. californicus</i>	Red to gray	—	—	0
<i>A. poolensis</i>	Green	—	—	0
<i>A. fradii</i>	Brown	—	—	0
<i>A. lipmanii</i>	Greenish	—	—	0
<i>A. viridochromogenus</i>	Dark brown	Dark	Scant white	0
<i>A. scabies</i>	Brownish to gray	Dark	—	0
<i>A. pheochromogenus</i>	Brown	—	—	0
215	Green	Dark gray	—	+
128	Green	—	White	+++
168	Brownish	—	White	+
<i>A. citreus</i>	Gray	—	—	0
<i>A. bovis</i>				+
<i>A. hominis</i>				++
<i>A. asteroides</i>				0
<i>A. madurae</i>				+++

The data brought out in table 4 tend to indicate that although some Actinomycetes show characteristic reactions upon blood agar, such as *A. griseus*, *A. madurae* and 128, the different species could not be readily differentiated by this medium alone. The cultural characters on blood agar can be used only to a very limited extent for the differentiation of these organisms. The interesting fact brought out in table 4 is the hemolysis of the blood in the blood agar, either as a result of the growth of the

organism or as a result of the production of an enzyme-like hemolysin. In the case of the organism producing hemolysis, a clear zone is formed around the colony on the plate or tube. In the case of *A. griseus*, *A. madurae* and *A. diastaticus* this zone was already observed in twenty-four hours.

Kuhn (1912) claimed that hemolysis is a step towards virulence, although not identical with virulence; the hemolysis of an organism is a sign of a higher step of adjustment of the organism to a parasitic (animal) existence. If this were the case for all microorganisms, the Actinomycetes isolated from the soil would be closely related to the parasitic Actinomycetes not only morphologically, but also physiologically which no doubt holds true, as will be seen throughout the work reported in these papers. But still another assumption can be made here. Several veterinarians expressed to the writer their opinion that, in certain localities, cattle feeding mainly on grass suffer quite often from outbreaks of actinomycotic diseases, and that this disease is obtained from the soil. Certain soils, as will be pointed out elsewhere, contain as many as 40 per cent Actinomycetes out of the total microbial population obtained by plate culture counts. The possibility of some of these Actinomycetes being able to produce animal diseases may be therefore not without foundation, although it has not been demonstrated as yet.

As to the chemical changes involved in the action of Actinomycetes upon blood, we might refer here to a paper by Baerthlein (1914) who stated that among the modifications which blood undergoes due to the action of bacteria, one can distinguish: (1) Hemolysis proper; the hemoglobin is set free and is not modified, the corpuscular stroma remains intact. (2) Hemoglobinopepsie; hemoglobin is set free and is completely digested, which brings about a discoloration of the medium, the corpuscular stroma remains intact. (3) Hemopepsie; the medium is decolorized as in the previous case, but the corpuscular stroma is digested as well as the hemoglobin. Cases 2 and 3 are observed only on solid media, while hemolysis proper is observed in liquid media. The use of the term "hemolysis" should therefore be

understood to include not only hemolysis proper, but also the digestion of the hemoglobin. Only hemoglobin, when heated or treated with acids, alkalies or various digestive enzymes, forms hematin, which has a brown appearance. It is possible that the action upon blood of those organisms, which do not produce any clear zone, but a brown pigment, is due to the fact that an enzyme is produced which converts the hemoglobin to hematin and related dark brown compounds, while the organisms forming a clear zone produce enzymes which split the hemoglobin molecule entirely liberating the iron or absorbing it. McNeal and Kahn (1918) have recently stated that hemolysis and proteolysis are probably two distinct phenomena. This is not borne out by the work on the Actinomycetes, since of the organisms that were isolated as saprophytes (from the soil) the ones that are most active proteolytically are also able to produce hemolysis of the blood in blood agar.

Growth of Actinomycetes on Loeffler's blood serum

Methods of inoculation and incubation were the same as for the blood agar. The results are reported in table 5.

Most of the Actinomycetes do not produce any characteristic growth on this medium, although all of them, with few exceptions, grew very well upon it. The liquefaction of the coagulated serum is an important biochemical property of some species of Actinomycetes. It will be noted that those organisms that were active in producing extensive hemolysis produced also liquefaction of the serum. These very strains were among the most active proteolytically, when grown on milk. It can thus be seen that the organisms that are able to decompose the casein of the milk, with the production of a strong proteolytic enzyme, also liquefy the blood serum, which is also a proteolytic phenomenon, and produce hemolysis of the blood in the blood serum.

To demonstrate that the liquefaction of the serum is a proteolytic phenomenon and is accompanied by a decomposition of the serum proteins, some of the liquefied serum was withdrawn from the culture and the amino nitrogen determined by the use

of the Van Slyke apparatus. *A. griseus* gave 18.10 mgm. amino nitrogen per 10 cc. and 128 gave 22.33 mgm. The serum originally contained 4.56 mgm. amino nitrogen per 10 cc. This indicates that the liquefaction of the serum was accompanied by the splitting of the serum proteins into peptones, polypeptides and probably amino acids and ammonia.

TABLE 5
The growth of Actinomycetes upon Loeffler's blood serum

NAME OF ORGANISM	COLOR OF GROWTH	SOLUBLE PIGMENT	AERIAL MYCELIUM	LIQUEFACTION TWO MONTHS
<i>A. violaceus-ruber</i>	Brown to red	Red	—	0
<i>A. violaceus-Caesari</i>	Gray	—	—	0
<i>A. albus</i>	Transparent	—	—	0
<i>A. griseus</i>	Creamy	—	White	++++
<i>A. diastaticus</i>	Transparent	—	—	++
<i>A. californicus</i>	Brown	—	—	0
<i>A. aureus</i>	Creamy	Black	—	0
<i>A. poolensis</i>	Creamy	—	—	0
<i>A. fradii</i>	Orange	—	—	0
<i>A. lavendulae</i>	White	Purple	—	0
<i>A. lipmanii</i>	Transparent	—	—	++
<i>A. viridochromogenus</i>	Brown	Black	White	0
<i>A. scabies</i>	Gray to somewhat yellowish	Brown	—	0
<i>A. rulgensis</i>	Gray	—	—	0
<i>A. reticuli</i>	Gray	Brown	—	0
<i>A. 120</i>	Gray	Brown	—	0
<i>A. b. bili</i>	Gray	Brown	—	0
<i>A. citreus</i>	Creamy	—	—	0
<i>A. bovis</i>	Yellowish	—	White	++
<i>A. hominus</i>	Transparent	—	—	+++
<i>A. asteroides</i>	Thin white	—	—	0
<i>A. madurac</i>	Yellowish	—	White	+++
128	Creamy	—	White	+++
168	Light brown	—	—	+
215	Sulfur-yellow	Black	—	0
206				++

SUMMARY

1. Blood agar forms a good medium for the growth of Actinomycetes. The production of a dark pigment by some and a clear zone, indicating hemolysis, by others is characteristic.

2. Loeffler's blood serum forms a good medium for the growth of Actinomycetes; the liquefaction of the serum by some and the production of a dark brown to black pigment by others are distinctive properties of some of these organisms.

3. The organisms that produce hemolysis and liquefy the blood serum are among the ones that can produce active proteolytic enzymes.

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STUDIES ON DIPHTHERIA TOXIN¹

II. THE RÔLE OF THE AMINO ACIDS IN THE METABOLISM OF BACTERIUM DIPHTHERIAE

LEWIS DAVIS AND NEWELL S. FERRY

From the Research Laboratory of Parke, Davis and Company, Detroit, Michigan

Received for publication, December 19, 1918

Attempts to cultivate *Bact. diphtheriae* in simple, non-protein media are recorded among the early studies with this organism. In 1892, Guinochet (1892) claimed to have grown diphtheria bacilli with toxin production in urine freed from albuminous constituents. Soon after, Uschinsky (1893) confirmed these findings and also reported the growth of *Bact. diphtheriae* with elaboration of toxin in a medium having ammonium lactate and sodium asparaginate as the sole sources of nitrogen. The toxin thus obtained gave positive Millon's and xanthoproteic reactions, was precipitable by alcohol, and in its other reactions indicated protein characteristics.

The view that diphtheria toxin is a form of protein seems to have been first advocated by Brieger and Fraenkel (1890). These authors considered it to be a peculiar kind of albumin, which, because of its toxicity, they called "toxalbumin." Wasserman and Proskauer (1891), while conceding the possibility that pure diphtheria toxin may be an albuminous body, were of the opinion that the chemical reactions of the above "toxalbumin" were probably due to albumoses, present as impurities derived from bouillon. They also agreed with the observations of Brieger and Fraenkel that, because of its great susceptibility, this "toxalbumin" of diphtheria contained only a small portion of actual diphtheria toxin in an undecomposed state.

¹ Read before the Society of American Bacteriologists, Baltimore, Md., December 28, 1918.

A unique view regarding the composition of diphtheria toxin was that advanced by Dzierzowski and Rekowski (1892). From the fact that no diphtheria toxin was formed until the culture became alkaline, these investigators concluded that diphtheria toxin was a combination of certain bases and albumoses, of a nature similar to alkali albuminates.

Brieger and Boer (1896) working with both diphtheria and tetanus toxins, claimed to have isolated a double zinc-toxin compound, which showed neither albumin nor peptone reactions, thus arguing against the protein character of diphtheria toxin. In a subsequent communication regarding his synthetic diphtheria toxin, Uschinsky (1897) did not consider it of albuminous nature, although Chamberland filtrates of diphtheria bacilli grown in his medium gave distinct protein reactions. Not all cultures of *Bact. diphtheriae* were found by him to be cultivable in a protein free medium, but when once accustomed to the medium, the organisms grew typically and as luxuriantly as in bouillon.

In an elaborate investigation comprehending seventy pure cultures of *Bact. diphtheriae*, Hadley (1907) was unable to obtain any noticeable growth on Uschinsky's medium. Continuing the experimentation, this investigator finally devised a medium containing glycocoll and ammonium lactate together with glycerin, sodium chloride, calcium chloride, magnesium sulphate and di-potassium phosphate. This he found not only permitted the growth of diphtheria bacilli, but produced a culture, which after sixteen days' incubation, killed guinea pigs in from thirty-six to thirty-eight hours. Cultures of *Bact. diphtheriae* which, at first, did not grow on the protein-free media, could be adapted to it by slow degrees and the solid-staining forms of the organism always manifested the most rapid and luxuriant growth both during and after the adaptation. Hadley further concluded that, of the three nitrogen bases employed in the study, urea seemed to be of slight value, while glycocoll gave the best growth and strongest toxin. Asparagin appeared to give better results than the urea although it was not as satisfactory as the glycocoll.

Hida (1908), in a paper concerning the significance of "pep-

tone" in the formation of diphtheria toxin concludes that the deutero-albumose portion of the peptone is the most important for this purpose, while heteroalbumose, protalbumose, and amphopeptone are subordinate factors. A subsequent article by the same author working with Teruuchi (1912-1913) also indicated that the production of toxin was influenced by the degree of decomposition of the "peptone." Most favorable results were furnished by a sample having 18 per cent of its total nitrogen in the monamino acid condition, 75 per cent, precipitable by tannin, and 30 per cent by a 65 per cent concentration of alcohol.

The chemical composition of diphtheria bacilli has been reported upon by Tamura (1914) who examined, washed and dried pellicles of the organisms cultivated in a 2 per cent peptone, sheep-kidney extract medium. A monoaminomonophosphatid and a lipid body were found to be present and the presence of adenin was considered as probable. Of the amino acids, arginin, *r* and *l* prolin, histidin, lysin, tyrosin, leucin, isoleucin, and valin were isolated, while tryptophane was demonstrated by reaction. On the other hand, no positive sulphur test could be obtained, and mycol was found to be absent.

More recently, Robinson and Rettger (1917, 1918) have reported upon the growth and toxin production of *Bact. diphtheriae* when cultivated in a medium containing the product "opsine" previously studied by Dalimier and Lancereaux (1913). According to their description, "opsine is a biuret-free product resulting from the combined action of trypsin, erepsin and pepsin on certain protein materials not named by the authors or manufacturers." It was found to be abiuretic, and to give a high formol titration for monamino acids, the latter including leucine, tyrosin and very little tryptophane. In addition to the opsine, Robinson and Rettger attempted the cultivation of *Bact. diphtheriae* in media composed of the protein-free acid hydrolysis products of casein, edestin, and lactalbumin.

Notwithstanding rapid and abundant growth of the organism, they found that very little diphtheria toxin was formed in the opsine, the minimum lethal dose being 0.2 per cent of the body weight. In the acid hydrolysis products media, the growth was

considerably slower and less luxuriant than in the opsin and there was almost no evidence of any toxin. Addition of fresh beef infusion to opsin caused even more profuse growth of the bacilli than in diphtheria bouillon, while the toxicity was increased twenty-fold over that of cultures in opsin only. These authors consider that the results of their experiments tend to disprove the theory advanced by Uschinsky (1897) and also by Hadley (1907) that diphtheria toxin can be directly synthesized from comparatively simple nitrogenous substances like the amino acids. On the contrary, they support the view "that more complex bodies, perhaps some of the proteoses, as claimed by Hida (1908), or polypeptids, are essential to the formation of toxin." What little toxin is found in protein-free media, according to these investigators, may be formed from the disintegration products of dead bacilli during the long period of incubation.

It will be readily conceded from the above resume that comparatively little information is available regarding the constitution of diphtheria toxin. The two experimenters, Hadley and Uschinsky, who claim bacterial synthesis of the toxin; injected cultures of *Bact. diphtheriae* grown in their non-protein media for determination of toxicogenicity. This technique is, at once, open to the criticism that, with diphtheria bacilli, pathogenicity toward susceptible animals is not necessarily an indication of toxicogenicity. It would seem more likely that the death of the test animals was produced by the living pathogenic organisms which survived the long incubation periods rather than by the disintegration products of dead bacilli as has been suggested by Robinson and Rettger.

Observations made by one of us, Davis (1917), in connection with a study of bacteriologic peptone, showed that, when incorporated in bouillon, the presence or absence of certain amino acids in the peptone had a decided influence on the growth of *Bact. diphtheriae* and the production of its toxin.

The purpose of the work involved in the present paper was to determine, if possible, by cultivation of diphtheria bacilli in media consisting principally of known amino acids, the rôle played by these important protein constituents in the nutritional and toxicogenic requirements of this organism.

EXPERIMENTAL PROTOCOLS

a. Preparation of materials

With the exception of sodium asparaginate, which was obtainable in sufficient purity, the amino acids and nitrogenous bases employed in the experimental media later described were individually prepared and their identity established. These bodies comprehended glycocoll, cystin, glutaminic acid hydrochloride, leucin, tyrosin, tryptophane, histidindichloride, glucoseamine hydrochloride, the purine bodies,—hypoxanthin and xanthin and the extractives,—creatin and creatinin.

Glycocoll was synthetically prepared, by the method of Nencki (1883), using powdered ammonium carbonate heated with monochloroacetic acid. The glycocoll was isolated as the copper salt, decomposed with hydrogen sulphide, clarified with purified animal charcoal and crystallized. Several recrystallizations gave pure needles, answering all requirements for glycine.

Washed wool was found to be a very satisfactory raw material for cystine. The procedure given by Folin (1910) was followed with some modifications. The wool was hydrolyzed with hydrochloric acid, an excess of sodium acetate added, the precipitate washed, and then redissolved in boiling 5 per cent hydrochloric acid. After clarification with purified bone black, an excess of ammonium hydroxide was added, the resultant phosphate precipitate removed, and the material precipitated by addition of acetic acid. Thorough washing and drying gave typical hexagonal plates of pure cystine.

Glutaminic acid was prepared as the hydrochloride from both gliadin and glutenin of wheat flour, according to the directions given by Osborne and Guest (1911). Hydrolysis was carried out with concentrated hydrochloric acid, the material decolorized, using in this case repurified blood charcoal, and the colorless filtrate finally concentrated to syrupy consistency in vacuum. On cooling for several days, a heavy crystalline deposit was obtained which was repurified from alcohol. The resultant product left no ash on ignition and showed all characteristics of glutaminic acid hydrochloride.

Both leucin and tyrosin were obtained from blood clots using the separation method of Habermann and Ehrenfeld (1902). The clots were hydrolyzed with sulphuric acid, (5 : 13) made alkaline with milk of lime, and the lime removed as calcium oxalate. The filtrate was then concentrated to obtain a maximum yield of crude crystals, and the leucin was separated from the tyrosin by boiling the crystals in a mixture of glacial acetic acid and alcohol. After hot filtration, the tyrosin was clarified with bone black and recrystallized several times from cold water. Typical needles of tyrosin, giving a very strong Millon's reaction, were obtained. Additional quantities of this amino acid were also derived from the preparation of tryptophane, as will be subsequently described.

The hot alcohol-acetic acid filtrate containing the leucin was treated with an excess of copper carbonate, boiled, filtered, and the dark blue filtrate concentrated to crystallization. The crystals were decomposed with hydrogen sulphide, the liquid decolorized with bone black and then again evaporated to the appearance of crystals. A number of recrystallizations from water furnished silver-like plates of pure leucin.

A modification of the method of Hopkins and Cole (1902) was employed for the preparation of tryptophane, using Bacteriologic Peptone, (Parke, Davis and Company) as the source of raw material, which separated quantities of nearly pure tyrosin, requiring only crystallization to be suitable for use. The filtrate, after separation of tyrosin, was made 5 per cent acid with sulphuric acid and precipitation was effected with the mercuric sulphate reagent.

After thorough washing with sulphuric acid (5 per cent), the precipitate was decomposed with hydrogen sulphide and the latter removed with carbon dioxide. A partial precipitation with the mercuric sulphate reagent to remove cystin was now made, the precipitate removed, and an excess of the reagent added to the filtrate. The precipitate was again washed, decomposed with hydrogen sulphide, the latter removed with carbon dioxide, and whatever sulphuric acid was present was quantitatively removed with the requisite amount of barium

hydroxide. Filtration gave a clear liquid, which was mixed with half its volume of 90 per cent alcohol, concentrated, decolorized with purified blood charcoal, and again concentrated in vacuum. After several crystallizations from alcohol, rhombic crystals of tryptophane were obtained giving an intense reaction with Benedict's (1909) modification of the glyoxilic acid reagent.

Blood clots were also found to be the most satisfactory source for the preparation of histidin. Essentially, the procedure given by Pauly (1904) was followed, the clots being hydrolyzed with concentrated hydrochloric acid. The hydrolysate was then concentrated, made only weakly acid with sodium carbonate, filtered, and then made distinctly alkaline with the soda. After freeing from ammonia and diluting, mercuric chloride was added to produce precipitation. The precipitate was thoroughly washed, dissolved in hydrochloric acid (1:4) made alkaline with soda, and diluted, which caused the whole of the precipitate to be again thrown down.

Decomposition of the precipitate, after washing, was effected by hydrogen sulphide, the liquid was clarified with bone black, filtered, and the filtrate evaporated to syrupy consistency. Equal volume of absolute alcohol was now added, and the material concentrated in vacuum. On cooling and recrystallizing small rhomboid crystals were obtained, giving an intense color with Pauly's (1904) reagent and answering all requirements for histidin dichloride.

The hydrochloride of glucosamine was prepared by hydrolysis of chitin, obtained from decalcified lobster shells. Hydrochloric acid (1:2) was employed as the hydrolyzing agent, the liquid was clarified with blood charcoal and the filtrate evaporated to the appearance of crystals. The latter were recrystallized several times from water and finally were obtained pure in the form of parallelograms. In aqueous solutions, they strongly reduce Fehling's solution, have a distinct acid reaction to litmus and, on boiling with sodium hydroxide, are decomposed with elimination of ammonia.

Beef extract was used as raw material for hypoxanthin, xanthin and creatin, following the method given in Hawk's Practical

Physiological Chemistry (1916). Neutral lead acetate was added to precipitate the inorganic constituents, the excess of lead was removed with hydrogen sulphide, and the clear filtrate was concentrated to a thick syrup. After standing in the refrigerator for forty-eight hours, the syrup was extracted with 88 per cent alcohol which left a residue of crystals. The latter were dissolved in water, decolorized with bone black, the filtrate concentrated to small volume and allowed to crystallize. Repeated crystallization from water gave a substance which was identified as pure creatin. Creatinin was prepared from the creatin by hydrolyzing with dilute sulphuric acid. The excess of the latter was removed with barium carbonate, the material was filtered and evaporated to dryness. Extraction of the residue with 95 per cent alcohol and evaporation of the solvent gave pure creatinin.

The alcoholic filtrate from the above creatin preparation was evaporated to remove the alcohol, made ammoniacal, and precipitation effected by ammoniacal silver nitrate. The precipitate was now treated with boiling nitric acid (specific gravity of 1.1) filtered, and the hypoxanthine silver nitrate (identified by its crystalline appearance) was allowed to separate. The latter was decomposed by hydrogen sulphide, filtered, concentrated to remove the hydrogen sulphide, and made alkaline with ammonia. Free ammonia was removed by heating, the precipitate filtered, and the filtrate concentrated to small volume. On standing for several days in the refrigerator, small colorless needles of hypoxanthine were obtained.

To the filtrate from the hypoxanthine silver nitrate, ammonia was added in excess, the resultant precipitate filtered off and then decomposed with hydrogen sulphide. The filtrate was evaporated to a syrup and allowed to crystallize. Several recrystallizations gave pure xanthin.

The final reaction of all of the media described in the succeeding paragraph was adjusted to come within the limits, $\text{CH}^+ = 1.0 \times 10^{-8}$ to $\text{CH}^+ = 7.0 \times 10^{-9}$. This range has been found by Davis (1918) in a previous study on diphtheria toxin, to give

very satisfactory results in the routine production of high potency toxin. It is important to note, in this connection that it was found necessary to use the colorimetric method for accurate values of the media reactions. Electrometric determination of the hydrogen ion concentration of adjusted media containing amino acids proved to be unreliable, due to a steady increase in hydrogen ion concentration. In a typical case, the value rose steadily from an initial $\text{CH}^+ = 9.5 \times 10^{-9}$ to about $\text{CH}^+ = 2.5 \times 10^{-7}$ at the end of ten minutes.

Clark and Lubs (1917), in their work on hydrogen ion concentration, mention that synthetic media present some special problems in reaction and suggest the possibility of decomposition on sterilization. From the fact that the same phenomenon occurred on adjusting an unheated amino acid medium, the heat action can, in this instance, be left out of consideration. It is very likely that the increase is due to some action of the hydrogen while bubbling through the synthetic medium.

In all of the experimental work discussed below, the organism employed was a toxicogenic strain of *Bact. diphtheriae* originally obtained from Dr. W. H. Park and known as "Park no. 8". When grown in regular plain bouillon, under proper conditions, this culture has repeatedly furnished a toxin of which one L+ dose is less than 0.15 cc. A twenty-four hour culture in this medium shows a heavy pellicle and deposit with the intermediate bouillon entirely clear.

Unless otherwise noted, the general technic for toxicogenicity tests consisted in cultivating the organism in the experimental medium, contained in large flasks for 14 days at 37°C. The purity of the culture was then checked, 0.4 per cent of purified cresols were added, the mixed material allowed to stand in the refrigerator for twenty-four hours, and then filtered through a Mandler filter. By this method a clear product was obtained for test in which any toxic effects due to bacterial protein were eliminated. The toxicity was determined by subcutaneous injection of 250 gram guinea-pigs in accordance with the usual procedure for valuation of the potency of diphtheria toxin.

b. Laboratory data

1. *Synthetic media.* For preliminary experimentation looking toward the utilization of amino acids for the growth and toxin production of *Bact. diphtheriae*, a number of synthetic media were prepared. The various ingredients comprising each preparation were dissolved in a stated volume of distilled water and heated in flowing steam for fifteen minutes. The requisite amount of 10 N NaOH was then added to give a final reaction in the completed product of $P_{\text{H}}^+ = 8.0$ to $P_{\text{H}}^+ = 8.2$. This was readily accomplished by cold titration against N/10 NaOH to a deep pink color with phenolphthalein, following the technic cited above (Davis) 1918.

The medium was now steamed again for fifteen minutes and the reaction checked colorimetrically (using phenolsulphonphthalein and standardized H_3BO_3 - KCl - NaOH solutions) as already discussed. After filtration and distribution as desired, the material was sterilized for twenty minutes at 115°C .

It was apparent, at once, that a *Bact. diphtheriae* culture accustomed to peptone bouillon could not be successfully cultivated directly in synthetic media without preliminary acclimatization. Accordingly, successive transfers were made from the bouillon into mixtures of bouillon and synthetic medium containing gradually increasing quantities of the synthetic media until finally what might be termed a "limiting concentration" of the synthetic medium was obtained. The various experimental media are discussed below in detail; and the general results are summarized in table 1.

Synthetic medium no. 1

CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER	CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER
	grams		grams
Tryptophane.....	0.30	Creatinin.....	0.10
Tyrosine.....	1.00	Sodium asparaginate.....	1.40
Leucine.....	3.00	Cystin.....	0.40
Glutaminic acid hydrochloride.....	1.60	Sodium chloride.....	4.00
Glycocoll.....	0.40	Dipotassium acid phosphate.....	3.00
Creatin.....	0.10		

Typical luxuriant growth was obtained in twenty-four hours with this medium in mixtures containing up to 96 per cent synthetic medium and 4 per cent bouillon. With concentrations of the synthetic medium greater than 96 per cent, there was a marked decrease in the luxuriancy of the growth and pellicle formation. Above 98 per cent of synthetic medium, practically no growth was discernible. Notwithstanding the heavy growth at 96 per cent, toxicogenicity tests made by the regular procedure at this concentration showed practically no toxin. Guinea-pigs receiving doses of 0.1 cc. showed only irritation at the point of injection.

Synthetic medium no. 2.

CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER	CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER
	<i>grams</i>		<i>grams</i>
Tryptophane.	0.40	Sodium asparaginate.	1.20
Tyrosin.	2.50	Histidindichloride.	0.30
Leucin.	2.50	Sodium chloride.	4.00
Glutaminic acid hydrochloride.	1.90	Dipotassium hydrogen phosphate.	3.00
Glycocoll.	0.85	Magnesium sulphate.	0.50
Creatin.	0.10	Potassium nitrate.	0.20
Creatinin.	0.10		
Cystin.	0.40		

Very satisfactory growth, but practically no toxin was obtained in mixtures containing 98 per cent of the above medium and 2 per cent of plain bouillon. With greater proportions of the synthetic medium the same effects were noted as with medium no. 1. Precipitation of the tyrosin in the medium under consideration, showed an excess of this constituent. The following preparation was accordingly devised to contain a diminished quantity of this amino acid and also as a new factor, glucosamine hydrochloride, to note any increased action through carbohydrate addition.

Synthetic medium no. 3

CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER	CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER
	<i>grams</i>		<i>grams</i>
Tryptophane	0.60	Histidindichloride.....	0.50
Tyrosin.....	0.80	Glucosaminehydrochloride.	1.50
Leucin.....	3.00	Sodium asparaginate.. . .	0.50
Glutaminicacidhydrochloride.	2.50	Sodium chloride.....	2.50
Cystin	0.40	Dipotassium acid phosphate.	3.00
Creatin.	0.20	Magnesium sulphate.....	0.40
Glycocoll.	0.80	Potassium nitrate.....	0.20

Practically the same results as regards growth and toxicogenicity were obtained with this preparation as with the preceding medium no. 2. The protocols of the foregoing experiments indicated that, in all probability, the preparation contained a number of superfluous constituents. Medium no. 4 was accordingly made up with only three amino acids and glucosaminehydrochloride, but with increased amounts of each of these substances.

Synthetic medium no. 4

CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER	CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER
	<i>grams</i>		<i>grams</i>
Tryptophane.....	1.25	Sodium chloride.....	2.50
Histidindichloride	1.25	Dipotassium acid phosphate.	3.00
Cystin.....	0.42	Magnesium sulphate.....	0.40
Glucosaminehydrochloride....	2.75		

Heavy typical growth was obtained in this case with 95 per cent of synthetic medium as the "limiting" concentration. With a greater proportion of synthetic medium than 97 per cent, almost no growth was obtained. As was expected, the toxicogenicity results were negative.

Synthetic medium no. 5

CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER	CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER
	<i>grams</i>		<i>grams</i>
Tryptophane.....	0.60	Creatinin..	0.08
Tyrosin.....	1.25	Xanthin.....	0.05
Leucin.....	3.00	Hypoxanthin.....	0.05
Glutaminic acid hydrochloride..	2.50	Sodium chloride.....	4.00
Glycocoll.....	0.85	Dipotassium acid phosphate..	3.00
Sodium asparaginate.....	1.00	Magnesium sulphate.....	0.50
Cystin.....	0.40	Glucose.....	1.50
Histidindichloride.....	0.50	Potassium nitrate. .	0.20
Creatin.....	0.20		

With this preparation, which was the most comprehensive synthetic medium investigated, heavy, typical growth was obtained in a mixture containing as high as 99.5 per cent of the synthetic and only 0.5 per cent of plain bouillon. A slight growth with slight pellicle formation was obtained even with 99.8 per cent of synthetic medium and only 0.2 per cent of plain bouillon, but with a concentration of bouillon less than the latter amount, the growth failed.

It was deemed of interest, in connection with this medium, to determine what proportions of the synthetic medium and bouillon gave maximum toxin production. It was found that a toxin having an L+ dose of 0.15 cc. was still obtained where the mixture contained 90 per cent synthetic medium and 10 per cent bouillon. At the "limiting" concentration of 99.5 per cent, however, even with an apparently maximum growth, potency valuations showed practically no elaboration of toxin, which corroborates previous findings with synthetic media.

By way of possibly obtaining some information as to the important constituents in the above medium, a number of mixtures were prepared containing 99 per cent of differing synthetic media together with 1 per cent of plain bouillon. Inoculation of each was made directly from cultures grown in a 99 per cent mixture of medium no. 5. The composition of the preparations is given below:

Synthetic medium no. 6

CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER	CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER
	<i>grams</i>		<i>grams</i>
Tryptophane.	1.60	Sodium chloride.....	2.50
Cystin.	0.40	Dipotassium acid phosphate.	3.00
Glutaminic acid hydrochloride.	2.50	Magnesium sulphate.....	0.40
Glucosamine hydrochloride....	1.50		

The transfers into a 99 per cent mixture of the above showed only a scant growth, indicating this to be an inferior medium to no. 5 for cultivating *Bact. diphtheriae*. On addition of sufficient bouillon to reduce the concentration of synthetic medium to 97 per cent, maximum intensity of growth was again restored.

Synthetic medium no. 7

CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER	CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER
	<i>grams</i>		<i>grams</i>
Tryptophane.	1.00	Dipotassium acid phosphate.	3.00
Cystin.	0.75	Magnesium sulphate.....	0.40
Sodium chloride.....	2.50		

Almost no growth was obtained here in a 99 per cent mixture, and only very scant growth with the bouillon concentration raised to 5 per cent. A reduction of the synthetic medium concentration to 90 per cent was found to be necessary before luxuriant, typical growth could be obtained.

Synthetic medium no. 8

CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER	CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER
	<i>grams</i>		<i>grams</i>
Tryptophane.....	1.60	Histidine hydrochloride.....	1.20
Cystin.....	0.40	Sodium chloride.....	2.50
Glutaminic acid hydrochloride.	2.50	Dipotassium acid phosphate.	3.00
Glucosamine hydrochloride...	1.00	Magnesium sulphate.....	0.40

Heavy typical growth, comparable in character and intensity to that obtained in dilutions of medium no. 5, was obtained in 99 per cent mixtures of the above preparation. The luxuriance in this case, however, began to diminish at 99.2 per cent and no growth was obtained in a mixture of 99.5 synthetic medium and 0.5 plain bouillon.

Medium no. 9. The fact that it was impossible to maintain metabolism of the microorganism in question on any of the preceding synthetic media alone, suggested that some important constituent might be lacking. By way of attacking the problem from a different angle, gelatine was hydrolyzed with 25 per cent sulphuric acid for twenty-four hours on the sand bath. Sufficient barium hydroxide was now added to make the hydrolysate distinctly alkaline and the resultant heavy precipitate was filtered off. The excess of barium hydroxide was next exactly neutralized with 10 per cent sulphuric acid, the precipitate removed and the clear filtrate (tested to prove absence of both Ba and SO_4 ions) was concentrated in vacuum to a thick syrup.

From the work of Levene and Beatty (1906), it appears that gelatin is lacking in cystin, tyrosine and tryptophane. To supply this deficiency in the above hydrolysate, the syrup was diluted with distilled water to a total solids concentration of 2 per cent and the following were added to each liter of medium:

CONSTITUENTS	AMOUNTS OF 1000 CC. OF DISTILLED WATER	CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER
	grams		grams
Tryptophane.....	0.40	Sodium chloride....	5.00
Tyrosine.....	1.50	Dipotassium acid phosphate	3.00
Cystin.....	0.40	Magnesium sulphate	0.50

The resultant product was prepared, distributed, and sterilized as already described, and acclimatization studies were carried out as with the straight, synthetic media. Contrary to expectations, both the macroscopical appearance and the morphological characteristics of the organisms under investigation indicated that this medium was inferior to most of the preceding

preparations. A mixture containing 90 per cent of the synthetic medium and 10 per cent of plain bouillon appears to be the "limiting" concentration permitting maximum growth and pellicle formation. Even at this dilution of the medium, the growth is not as vigorous and the formation of pellicle appears more scanty than obtained in the unmodified preparations. Corroborating the foregoing, toxicogenicity results were negative.

TABLE 1
Growth and toxicogenicity of Bact. diphtheriae in synthetic media

MEDIUM NUMBER	MAXIMUM GROWTH CONCENTRATION		TOXICOGEN- ICITY RESULTS	REMARKS
	Synthetic	Bouillon		
	<i>per cent</i>	<i>per cent</i>		
1	96.0	4.0	Negative	Organisms shorter, stain solid
2	98.0	2.0	Negative	Appearance more typical
3	98.0	2.0	Negative	Same appearance as no. 2
4	95.0	5.0	Negative	Growth not as heavy as preceding
5	99.5	0.5	Negative	Toxin having L+ dose = 0.15 cc obtained with 90 per cent synthetic, 10 per cent bouillon
6	97.0	3.0	Negative	Heavy growth light pellicle formation
7	90.0	10.0	Negative	Short, plump bacilli, stain solid
8	99.0	1.0	Negative	Heavy, typical growth and pellicle formation
9	92.0	8.0	Negative	Inferior growth scanty pellicle
10	90.0	10.0	Negative	Same as preceding

Medium no. 10. The fact that gelatine is of animal origin suggested the use for a culture medium of a vegetable protein hydrolysate. For this purpose, gliadin, the prolamine obtained from wheat, was prepared, according to the procedure given by Osborne and Clapp (1907). These investigators have shown that lysine is absent in gliadin, and that this protein contains only 0.02 per cent of glycocoll.

The gliadin was hydrolyzed exactly as carried out with the gelatin, using both the 25 per cent sulphuric acid and the barium hydroxide, and the final product, containing neither Ba nor SO₄ ions, was also diluted to a final total solids content of 2 per cent. Equal parts of this hydrolyzed product and of the fin-

ished medium no. 9 were then taken, 0.2 gram of tryptophane added per liter, the reaction adjusted, and this composite was then finished in the usual way. As will be noted from Table 1, practically the same results were obtained with this experimental preparation as with medium no. 9, and here, also, toxin production was unsuccessful.

2. *Elimination media.* It appears to be clearly indicated from the preceding experiments that metabolism of *Bact. diphtheriae* is not permitted in a medium composed principally of amino acids. That the amino acids are, however, important factors in both growth and toxin elaboration with this organism seems to be plainly evidenced from the results already detailed with medium no. 5. The latter have also demonstrated that not all of these protein degradation products can exercise the same influence.

Preliminary cultivation in plain beef infusion carried out with the *Bact. diphtheriae* strain employed above, showed that only a scant growth with no formation of toxin is possible in this medium. A Liebig's Extract of Beef solution gave even poorer results. More luxuriant growth but still no toxicogenicity occurred in a 2 per cent solution of peptone.

With the preceding facts in mind, several series, of what might be termed "elimination" media, were now devised having in each series one or two of the above three substances as basic ingredients. The individual amino acids or the other materials incorporated in the synthetic media already investigated were then added in the same quantities as previously employed. Adjustment of the reaction and finishing of these media were in accordance with the usual technic. In order to acclimatize the diphtheria culture to the various media, the organisms in each case were cultivated first for three successive generations in "starter" flasks containing 30 cc. of the special broths and then inoculated into the large flasks.

The experimental preparations, together with the results obtained, are detailed in the accompanying table 2. As may be noted from the table, five series of the "elimination" media were prepared. The first set contained plain beef infusion as the

basic ingredient, the second had 2 per cent peptone (Bacteriologic, Parke, Davis and Company) solution, and for the third series, a 1 per cent Liebig's Extract of Beef solution served as the common constituent. In the remaining two sets, ordinary bouillon (2 per cent peptone, beef infusion and 0.5 per cent of sodium chloride) and a meat extract bouillon (2 per cent peptone, 0.5 per cent salt, 1 per cent Liebig's Extract) were used as basic constituents.

The presence of any of these basic media in a preparation is indicated in the table by a (*) and the absence of the others, by the symbol (-). The intensity of the growth has been characterized as follows: (++++) = heavy; (++++) = good; (++) = moderate, and (+) = scant. In the valuation of toxin potency, it was not deemed necessary to determine the minimum lethal dose (M. L. D.) above 1 cc., but where such results were obtained they have been designated by "M. L. D. = 1 cc. +."

Probably the most significant of the data presented in table 2 are the results furnished with beef infusion alone, as a basic medium. Where 2 per cent peptone solution served as the common ingredient, practically no differences could be distinguished on addition of the special constituents. In each case, only the moderate growth due to peptone itself was obtained. Except where cystin was added, the results given with Liebig's Extract were negative.

The purpose in using regular (peptone beef infusion) bouillon and Liebig's Extract (peptone) bouillon as basic media was to determine whether addition of the amino acids and other special constituents caused any increase in potency of the toxin. As may be noted from table 2, the final toxicities furnished by the plain bouillon, special combinations are no higher than those given by the same culture of *Bact. diphtheriae* when cultivated in the plain bouillon alone. Confirming expectations, the special media containing Liebig's Extract gave results uniformly inferior to beef infusion and, in no case, a final toxin of which, one L+ dose was less than 1 cc.

TABLE 2

Effect of special constituents on growth and toxicogenicity of Bact. diphtheriae

CONCENTRATION OF SPECIAL CONSTITUENTS IN 1000 cc.	BASIC MEDIA			CHARACTER OF GROWTH	POTENCY OF TOXIN	REMARKS
	Beef infusion	2 per cent peptone	1 per cent Liebig's extract			
Tryptophane, 0.60 gram	*	—	—	++++	M. L. D. = 0.01 cc.	Strong growth with beef infusion base. L + dose of toxin = 0.75 cc.
	—	*	—	++	M. L. D. = 1 cc. +	
	—	—	*	+	M. L. D. = 1 cc. +	
	*	*	—	++++	L + = 0.15 cc.	
	—	*	*	+++	L + = 1 cc.	
Cystin, 0.50 gram	*	—	—	++++	M. L. D. = 0.0075	L + dose of toxin with been infusion = 0.5 cc.
	—	*	—	++	M. L. D. = 1 cc.	
	—	—	*	+++	M. L. D. = 1 cc.	
	*	*	—	++++	L + = 0.15 cc.	
	—	*	*	+++	L + = 1 cc.	
Tyrosin, 1.25 grams	*	—	—	++	M. L. D. = 1 cc. +	Only moderate growth with beef infusion base
	—	*	—	++	M. L. D. = 1 cc. +	
	—	—	*	+	M. L. D. = 1 cc. +	
	*	*	—	++++	L + = 0.15 cc.	
	—	*	*	+++	L + = 1 cc.	
Glutaminic acid hydrochloride, 2.50 grams	*	—	—	++++	M. L. D. = 0.05 cc.	Growth with beef infusion not as heavy as with cystin
	—	*	—	++	M. L. D. = 1 cc. +	
	—	—	*	+	M. L. D. = 1 cc. +	
	*	*	—	++++	L + = 0.15 cc.	
	—	*	*	+++	L + = 1 cc.	
Histidin di-chloride, 0.50 gram	*	—	—	+++	M. L. D. = 1 cc. +	Growth with beef infusion base comparable to tyrosine
	—	*	—	++	M. L. D. = 1 cc. +	
	—	—	*	++	M. L. D. = 1 cc. +	
	*	*	—	++++	L + = 0.15 cc.	
	—	*	*	+++	L + = 1 cc.	
Leucin, 3.00 grams	*	—	—	++	M. L. D. = 1 cc. +	Moderate growth only with beef infusion base
	—	*	—	++	M. L. D. = 1 cc. +	
	—	—	*	+	M. L. D. = 1 cc. +	
	*	*	—	++++	L + = 0.15 cc.	
	—	*	*	+++	L + = 1 cc.	
Glycocoll, 0.75 grams	*	—	—	++++	M. L. D. = 0.07 cc.	Beef infusion growth not as heavy as with glutacid HCl
	—	*	—	++	M. L. D. = 1 cc. +	
	—	—	*	+	M. L. D. = 1 cc. +	
	*	*	—	++++	L + = 0.15 cc.	
	—	*	*	+++	L + = 1 cc.	

TABLE 2—Continued

CONCENTRATION OF SPECIAL CONSTITUENTS IN 1000 cc.	BASIC MEDIA			CHARACTER OF GROWTH	POTENCY OF TOXIN	REMARKS
	Beef infusion	2 per cent peptone	1 per cent Liebig's extract			
Sodium asparaginate 1.5 grams	*	—	—	++++	M. L. D. = 0.05 cc.	Behavior same as with glutaminic acid hydrochloride
	—	*	—	++	M. L. D. = 1 cc. +	
	—	—	*	+	M. L. D. = 1 cc. +	
	*	*	—	++++	L + = 0.15 cc.	
	—	*	*	++++	L + = 1 cc.	
Creatin, 0.2 gram; creatinin, 0.15 gram	*	—	—	++++	M. L. D. = 1 cc. +	Scant growth with beef infusion base
	—	*	—	++	M. L. D. = 1 cc. +	
	—	—	*	+	M. L. D. = 1 cc. +	
	*	*	—	++++	L + = 0.15 cc.	
	—	*	*	++++	L + = 1 cc.	
Xanthin, 0.05 gram; hypoxanthin, 0.05 gram	*	—	—	+	M. L. D. = 1 cc. +	Beef infusion growth same as preceding
	—	*	—	++	M. L. D. = 1 cc. +	
	—	—	*	+	M. L. D. = 1 cc. +	
	*	*	—	++++	L + = 0.15 cc.	
	—	*	*	++++	L + = 1 cc.	
Glucose	*	—	—	+	M. L. D. = 1 cc. +	Beef infusion growth same as with creatin
	—	*	—	++	M. L. D. = 1 cc. +	
	—	—	*	+	M. L. D. = 1 cc. +	
	*	*	—	++++	L + = 0.25 cc.	
	—	*	*	++++	L + = 1.5 cc.	
Glucoseamine HCl, 2.00 grams	*	—	—	+	M. L. D. = 1 cc. +	Behavior same as with glucose
	—	*	—	++	M. L. D. = 1 cc. +	
	—	—	*	+	M. L. D. = 1 cc. +	
	*	*	—	++++	L + = 0.2 cc.	
	—	*	*	++++	L + = 1.5 cc.	
Control	*	—	—	+	M. L. D. = 1 cc. +	Behavior same as preceding
	—	*	—	++	M. L. D. = 1 cc. +	
	—	—	*	+	M. L. D. = 1 cc. +	
	*	*	—	++++	L + = 0.15 cc.	
	—	*	*	++++	L + = 1 cc.	
NaCl, 4 grams; K ₂ HPO ₄ , 3 grams; MgSO ₄ , 0.4 grams; KNO ₃ , 0.2 grams	*	—	—	+	M. L. D. = 1 cc. +	Behavior same as preceding
	—	*	—	++	M. L. D. = 1 cc. +	
	—	—	*	+	M. L. D. = 1 cc. +	
	*	*	—	++++	L + = 0.15 cc.	
	—	*	*	++++	L + = 1 cc.	

The addition of cystin to plain beef infusion as may be seen from table 2 not only permits heavy typical growth with *Bact. diphtheriae*, but a relatively strong toxin is also elaborated. Tryptophane, similarly, allows a heavy growth, but the resultant toxin is not as active as obtained with cystin. Practically the same results were obtained on adding either glutaminic acid hydrochloride or sodium asparaginate to the beef infusion. *Bact. diphtheriae* grew luxuriantly in both cases, yielding a final toxin, however, which had only one-fifth the strength of that obtained with tryptophane. The growth in presence of glyco-coll, it will be noted, was not as heavy as with the two preceding constituents, and the filtered toxin was also weaker (minimum lethal dose equals 0.07 cc.).

Tyrosin, leucin, and histidindichloride, when added to beef infusion, each appear to yield the same results. They permit a moderate growth of the organism, but no toxin of consequence is elaborated. Comparison of the protocols given by the remainder of the special constituents investigated, with those furnished by the control preparations shows no essential difference. These include the results obtained with creatin and creatinin, xanthin and hypoxanthin, the hydrochloride of glucosamine, glucose, the inorganic ingredients, sodium chloride, magnesium sulphate, potassium nitrate and dipotassium hydrogen phosphate. The growth of the bacillus and formation of toxin in the various "control" media have already been discussed.

DISCUSSION

Notwithstanding the fact that the amino acids by themselves appear to be unable to support the metabolism of *Bact. diphtheriae*, some of these protein degradation products, as has been shown in the foregoing, have a decided influence on growth and toxin production with this organism. Of the various amino acids investigated, cystin seems to be of special importance. Addition of this sulphur containing body not only permits heavy typical growth of the diphtheria bacillus in a medium like beef infusion which is merely capable of maintaining metabolism of *Bact. diphtheriae*, but simultaneously the organism is now able to

elaborate a potent toxin. That this toxin is a true diphtheria toxin is amply demonstrated by its ability to neutralize diphtheria antitoxin giving an L+ value for potency closely corresponding with the theoretical value derived from determination of the minimum lethal dose.

The fact that tryptophane in beef infusion allows a growth of *Bact. diphtheriae* practically of the same intensity as does cystin, while the resultant toxin is of inferior potency, would suggest that the sulphur complex in the cystin molecule enters into toxin production. It seems likely that diphtheria toxin contains sulphur and that where tryptophane and other non sulphur containing amino acids permit toxin elaboration, with beef infusion, the sulphur portion of the toxin molecule is derived from the infusion. Indol tests made after growth of the organism in tryptophane containing media were found to be negative, indicating that in the utilization of this compound by the diphtheria bacillus, there is no intermediate separation of the indol ring.

The very close relationship of asparaginic acid (amino succinic acid) to its next higher homologue, glutaminic acid (amino glutaric acid) appears to be closely paralleled by the similarity in the protocols furnished by both glutaminic acid hydrochloride and sodium asparaginate. The growth of the organism is of equal intensity in presence of either of these substances and a toxin of practically the same potency is elaborated in both cases.

It is worthy of note that glycocoll added to beef infusion with a reaction adjusted to the proper H ion concentration permits of heavy growth and toxin elaboration with *Bact. diphtheriae*. Since glycocoll has no asymmetric carbon atom, it might be deduced that the major factor contributing to formation of toxin in this case need not necessarily be optically active. Furthermore, although equivalent results were obtained with both sodium asparaginate and glutaminic acid hydrochloride, the glutaminic acid is dextro rotatory while the asparaginic acid turns the plane of polarized light to the left. Seemingly, the direction of rotation is also of minor consequence.

The fact that it was found impossible to grow *Bact. diphtheriae* satisfactorily and obtain a production of toxin except in the

presence of beef infusion, even though the quantity of the latter was relatively very small, suggests a food hormone requirement for growth, and particularly for toxin production, by the organism. Davis (1917) has discussed the question of food accessory factors with reference to cultures of hemophilic bacilli and both Lloyd (1917) and Drew (1917) with the meningococcus. They all conclude that the presence of these factors is essential. Assuming that both peptone and Liebig's Extract of beef are deficient in vitamins, and that ordinary beef infusion has these factors present, this may partially explain the scant growth and absence of toxin which obtained in presence of both the peptone and the extract.

It is readily appreciated that the preceding experimentation has not directly included other important amino acids, particularly proline and lysine, although these have been comprehended in the hydrolysis media nos. 9 and 10 detailed in table 1. The experimental data obtained, however, warrant the belief that it is not possible to maintain metabolism of *Bact. diphtheriae* in a medium composed entirely of amino acids, even after addition of muscle extractives and mineral salts.² This lends strength to the theory that diphtheria toxin is an excretory product formed by the organism only when certain amino acids and accessory factors, the latter perhaps of a vitamin character, are present. It does not seem likely, as has been claimed by several, that the toxin is a synthetic product, built up directly by *Bact. diphtheriae* from mineral salts and nitrogen compounds of the character of amino acids.

SUMMARY

1. *Bact. diphtheriae* could not be cultivated in synthetic media composed of amino acids and mineral salts adjusted to the optimum H ion concentration. Addition of the extractives creatin and creatinin and the purine bases, xanthin and hypoxanthin, was of no advantage.

² Experimentation in progress since this paper was read indicates that growth of *Bact. diphtheriae* in a 100 per cent synthetic medium is possible. This will be discussed in a later publication.

2. Typical luxuriant growth of *Bact. diphtheriae* was obtained in a mixture of 99.5 per cent synthetic medium and only 0.5 per cent of bouillon. Production of active toxin, however, required the presence of 10 per cent bouillon.

3. Cystin, when added to plain beef infusion, a culture medium, just capable of maintaining growth of *Bact. diphtheriae*, not only permitted rapid and heavy vegetation, but a strong toxin (minimum lethal dose equals 0.0075 cc.) was also elaborated. This appears to be a true toxin capable of neutralizing the corresponding diphtheria antitoxin.

Tryptophane, under the same conditions, allowed almost as heavy growth, but the resultant toxin (minimum lethal dose equals 0.01 cc.) was weaker in potency. Glutaminic acid hydrochloride and sodium asparaginate gave parallel results. The growths were not as heavy as with cystin or tryptophane and, in both cases, the toxin produced had only one-fifth of the strength (minimum lethal dose equals 0.05 cc.). Heavy typical growth was also obtained with glycocoll, but the toxin elaborated (minimum lethal dose equals 0.07 cc.) was much less potent.

Moderate growth and practically no toxin production by *Bact. diphtheriae* were obtained in beef infusion containing leucin, tyrosin, or histidindichloride. Addition to beef infusion of any of the following: creatin and creatinin, xanthin and hypoxanthin, glucose, glucoseamine hydrochloride, and the inorganic salts, sodium chloride, dipotassium hydrogen phosphate, magnesium sulphate, and potassium nitrate permitted only scant growth and consequently no toxin. Substitution of the beef infusion by either peptone or Liebig's Extract resulted in deficient growth and toxin formation. This, and the data furnished by the synthetic media suggest a vitamine requirement not only for luxuriant growth of the organism but particularly for strong toxin production.

4. The results obtained favor the belief that diphtheria toxin is not a synthetic product, but rather a catabolic substance elaborated by *Bact. diphtheriae* only in presence of certain amino acids and accessory factors, the latter probably of a vitamine character.

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THE INTERPRETATION OF *B. COLI* TEST RESULTS ON A NUMERICAL AND COMPARATIVE BASIS

AS APPLIED TO THE INVESTIGATION OF WATER AND SEWAGE

MILTON F. STEIN

Received for publication, January 30, 1919

One of the principal shortcomings of the *B. coli* test lies in the interpretation of the results obtained. A statement of the number of colon bacilli present in the liquor tested is the only presentation of results which would be intelligible to the layman, and an analysis of professional discussions as to the interpretation of *B. coli* tests for specific purposes will often disclose that one of the things befogging the issue is the failure to agree on what a certain percentage of positive tests really means.

By use of the theory of probabilities, it is possible to interpret the results as usually obtained in terms of definite numerical values. The writer devised a method for doing this several years ago, of which a synopsis was published in the Engineering News-Record of May 25, 1917. Various queries and comments relative thereto have prompted the preparation of this article. For purposes of identification this method of interpreting *B. coli* results will be referred to as the *B. coli* factor method.

The principal objects of this article are:

1. To show the rationality of the *B. coli* factor method by developing the same from a few universally accepted elementary conceptions in probabilities.
2. To discuss the errors to which the *B. coli* test is subject and to show how these errors may be evaluated.
3. To present in the form of a chart the necessary data from which, given a certain percentage of positive results in a series of tests, the number of *B. coli* per cubic centimeter and the expected errors in the result can be obtained at once, without computation,

thereby making this method available to those with a limited mathematical training and without the consumption of the time required for computation by methods now in vogue.

4. To propose a means for handling the troublesome question of variation in *B. coli* content from test to test and its effect upon average values.

The author believes the method here given to be rationally sound, practicable, and easy of application. He wishes to give warning, however, that with this, and with any method that might be devised, the reliability of results depends upon a sufficiency of tests, and upon the alertness and good judgment of the analyst in accommodating his samples: in size, to the variable *B. coli* content of the water; and in time, to the fluctuations therein. This method offers means of assisting in the formation of judgment on these factors, and avoids the compounding of tests of different degrees of accuracy, which leads to confusing and irrational results.

MATHEMATICS OF THE *B. COLI* FACTOR

The basis of this method for expressing numerically the results of a series of *B. coli* tests is the mathematical theory of probabilities. Moreover, it can be developed directly from the fundamental conception of probability as used in that theory. This is often illustrated by the following example; if a bag contains nine white balls and one black ball and a ball is withdrawn at random, the probability that it is black is one-tenth. Stated as a general case: if of t equally likely occurrences, f are favorable to a certain event E , then the probability of its occurrence is given by the proper fraction $\frac{f}{t}$, or

$$P(E) = \frac{f}{t} = p$$

Since one of the t occurrences is certain to happen, the fraction representing the probability in this case being $\frac{t}{t}$ or 1, 1 is the

mathematical representation of certainty. It then follows that the probability of the non-occurrence of the event E is $1 - p$ or

$$P(\bar{E}) = 1 - p = q$$

This conception of the probability of an event is confirmed by logicians and philosophers, and is accepted by common usage, as in games of chance and in discussing the possible success or failure of an enterprise, when it is common to say that it has one chance in a certain number of succeeding, although the fraction so chosen is usually a guess, pure and simple. So that the starting point in the development of this method is a universally accepted axiom based largely on common experience.

The next step relates to a series of trials. If, instead of one trial, as above, s successive trials are made, with the understanding that the ratio of favorable cases, or p , does not change during the trials (which means in the white and black ball scheme, that the balls withdrawn are replaced after each trial or else that the total number is so large that the ratio is not appreciably affected by the withdrawals during the series of trials) then the probabilities of the favorable event occurring s , $s - 1$, $s - 2$, . . . 0 times are given by the values of the terms of the expansion of $(p + q)^s$ of which s , $s - 1$, $s - 2$, etc., are the exponents of p . While this is susceptible of exact proof, such proof would be too lengthy for this discussion. However, an example may serve to show the manner of its derivation. Suppose a bag containing 10 per cent black balls, the rest being white, and suppose four successive drawings to be made (with replacements). Then $p = 0.1$, $q = 0.9$, $s = 4$, and the probabilities of obtaining 4, 3, 2, 1 or 0 black balls are given by the terms of the expansion $(0.1 + 0.9)^4$ which by the binomial theorem are:

$$0.1^4 + 4 \times 0.1^3 \times 0.9 + 6 \times 0.1^2 \times 0.9^2 + 4 \times 0.1 \times 0.9^3 + 0.9^4$$

Evaluating these terms:

Probability of obtaining 4 black balls	= 0.0001
Probability of obtaining 3 black balls	= 0.0036
Probability of obtaining 2 black balls	= 0.0486
Probability of obtaining 1 black ball	= 0.2916
Probability of obtaining 0 black balls	= 0.6561
Total = Certainty	= 1.0000

In the first term, which gives the probability of drawing four black balls, it is evident that there are nine chances of drawing a white ball, and one chance of drawing a black ball at the first trial. Anyone of these ten balls having been drawn, anyone of ten other balls may be drawn on the second trial, giving ten possible combinations with the ball first drawn, or 100 possible combinations with the ten balls anyone of which may have been drawn on the first trial. Similarly any one of ten balls may be drawn at the third trial with each of the 100 possible combinations of the first two trials giving 1000 combinations, and at the fourth trial any one of ten with the 1000 combinations of the first three trials, giving a total of 10,000 possible combinations for the four trials. Of these, there is only one possible combination for drawing four black balls, or one chance in ten thousand giving by the definition:

$$P_4 = 1 \div 10,000 = 0.0001$$

In the second term, which gives the probability of drawing three black and one white ball, and in each of the others, there are evidently also 10,000 possible combinations of four balls to be obtained in the four trials. Assuming that it is desired to draw first three black balls and then a white ball in succession, there is one chance in a thousand of obtaining the one possible combination of three black balls in the total 1000 possible combinations of the first three trials. If this rare combination is obtained, there are evidently nine chances out of ten for obtaining a white ball at the fourth drawing, so that there are nine combinations of the possible 10,000 which give three black balls and one white ball in the desired sequence. If the order of drawing is immaterial, the following arrangements may be obtained:

	1	2	3	4
First drawing...	●	●	●	○
Second drawing...	●	●	○	●
Third drawing.....	●	○	●	●
Fourth drawing.....	○	●	●	●

thus giving a total probability of

$$p_3 = 4 \times 9 \div 10,000 = 0.0036$$

In the third term, which gives the probability of drawing two black and two white balls, there is one chance in 100 of drawing two black balls in the first two trials. Assuming this to have been accomplished there are nine chances out of ten of drawing a white ball at the third trial, and with each of these nine there are nine additional chances of drawing a white ball at the fourth trial giving a total of $1 \times 9 \times 9$ or 81 chances in the 10,000 possible ones of attaining the desired result in the sequence given. The possible sequences of the two white and two black balls are:

	1	2	3	4	5	6
First drawing.....	●	○	○	●	○	●
Second drawing....	●	●	○	○	●	○
Third drawing.*..	○	●	●	●	○	○
Fourth drawing. . .	○	○	●	○	●	●

thus giving a total probability of

$$p_2 = 6 \times 81 \div 10,000 = 0.0486$$

In the fourth term, which gives the probability of drawing one black and three white balls, there is one chance in ten of drawing a black ball at the first trial, nine chances in ten of drawing a white ball at the second trial, at the third trial and at the fourth trial, and there are four possible orders in which one black and three white balls may be drawn in the four trials, so that the reasoning is the same as in the second term, with p and q reversed, however.

The fifth term is derived by reasoning analogous with that used in the first term.

This rather lengthy example shows that there are no abstruse manipulations or dubious assumptions in finding the probabilities in a series of trials, but that this involves merely finding the number of combinations favorable to an event and

the total number of possible combinations and then applying the fundamental definition of probability as a ratio of the two.

Now, in the *B. coli* test, a definite quantity of the liquid to be examined is placed in a fermentation tube containing media from which the colon bacilli, if present can produce gas, which being trapped in the closed end of the tube, indicates their presence. If we ascertain the volume of such a bacillus, and divide a comparatively large volume of the sample by this volume we can obtain the number of droplets of liquid equivalent in size to the colon bacillus. Assuming that we know by some means, the number of colon bacilli in the sample, we can consider these as black balls, and the water droplets of equal size as white balls. Then the ratio of the number of coli to the total number of droplets gives the value of p or the probability of obtaining a colon bacillus by withdrawing one droplet from the sample.

Let us suppose that the amount used in the test (0.1, 1, 10 cc.) contains s droplets, then the probabilities of obtaining s , $s - 1$, $s - 2$, etc., coli would be equal to the terms of the expansion $(p + q)^s$ as already explained above. However, we only know from the presence or absence of gas in the test, whether one or more *B. coli* are present, or whether they are entirely absent. The question then is, how, from a number of tests, some positive and some negative, we can deduce the most probable number of *B. coli* per cubic centimeter. The last term of the expansion — q^s — gives the probability of withdrawing no *B. coli* with the quantity taken for test, therefore $1 - q^s$ must give the total probability of obtaining one or more *B. coli* in the quantity tested, or the probability of obtaining a gas indication in the test. From one test no deduction can be drawn, but in a number of tests, the ratio of the positive results to the total number will approximate this probability of $1 - q^s$, which being designated by P , we may write the following:

$$\lim_{N = \infty} P = 1 - q^s \quad (I)$$

Where N is the number of tests made.

The greater the number of tests, the more closely will the ratio of positive to total tests approach this value of P , the accuracy increasing as the square root of the number of tests.

The use of equation I is tedious due to the necessity of reducing the sample to droplets the size of bacteria (of which there would be about 500,000,000,000 per cubic centimeter) resulting in numbers too large to handle, besides involving an element of doubt as to the actual size of the bacteria. Larger particles would serve equally well, distinguishing between those containing *B. coli* and those not containing any. If taken too large, the results obtained would be incorrect, since this would reduce the number of particles too much, as the theory of probabilities assumes the use of large numbers. This difficulty can however be entirely surmounted and all doubt as to the size of particles and uncertainty of results caused thereby eliminated as follows: Let

Q = the proportion of negative tests obtained, so that

$$Q = 1 - P$$

m = the number of units containing one *B. coli*, so that

$$\frac{1}{m} = p; \quad 1 - \frac{1}{m} = q$$

xm = number of units tested (corresponding to s as used above) x being any whole number or fraction, and representing the number of coli in the sample tested.

Then

$$Q = q^s = \left(1 - \frac{1}{m}\right)^{xm}$$

Letting $x = 1$, $Q = \left(1 - \frac{1}{m}\right)^m$

Expanding this by the binomial theorem:

$$\begin{aligned} \left(1 - \frac{1}{m}\right)^m &= 1^m - m \cdot 1^{m-1} \cdot \frac{1}{m} + \frac{m(m-1)}{2} \cdot 1^{m-2} \cdot \frac{1}{m^2} - \\ &\quad \frac{m(m-1)(m-2)}{2 \cdot 3} \cdot 1^{m-3} \cdot \frac{1}{m^3}, \text{ etc.} \end{aligned} \quad (\text{A})$$

The first two terms, each equal to 1, cancel, the remaining terms being multiplied out, and reduced to a common denominator give, approximately:

$$\left(1 - \frac{1}{m}\right)^m = .3678 \text{ etc.} - \frac{1}{6m} - \frac{1}{6m^2} + \frac{1}{6m^3} - \frac{1}{5m^4}, \text{ etc.} \quad (\text{B})$$

By making m sufficiently large, all the terms following the first may be made as small as desired, and so can be neglected.

It can be shown that the exact value of the first term of this modified series is, when evaluating the first $n + 2$ terms of series A:

$$\text{First term} = \frac{1}{2!} - \frac{1}{3!} + \frac{1}{4!} - \frac{1}{5!} + \frac{1}{6!} - \frac{1}{7!} \cdots = \frac{1}{(n+1)!}^*$$

Evaluating the first eight terms of this series gives

$$\lim \left(1 - \frac{1}{m}\right)^m = 0.367879 \text{ (correct to 5 places)}$$

$$m = \infty$$

*The $(n+2)$ 'd term of series A contains $n+1$ factors in the numerator, each involving m , which by multiplication give an expression the first term of which is m^{n+1} . The denominator of the $(n+2)$ 'd term is $(n+1)! m^{n+1}$. So that this term may be written

$$\frac{m^{n+1} + am^n + bm^{n-1} + cm^{n-2} \dots, \text{ etc.}}{(n+1)! m^{n+1}}$$

or

$$\frac{1}{(n+1)!} + \frac{a}{(n+1)!m} + \frac{b}{(n+1)!m^2} + \frac{c}{(n+1)!m^3} + \dots, \text{ etc.}$$

The $(n+1)$ 'st term, similarly may be written

$$\frac{m^n + gm^{n-1} + hm^{n-2} + im^{n-3} + \dots, \text{ etc.}}{n! \cdot m^n}$$

or

$$\frac{1}{n!} + \frac{g}{n!m} + \frac{h}{n!m^2} + \frac{i}{n!m^3} + \dots, \text{ etc.}$$

and so on for the other terms, a, b, c, g, h, i —are unknown constants, which may be positive or negative.

Summing up all the terms in which the variable m is absent as the first term of equation B, those containing $\frac{1}{m}$ as the second term, those containing $\frac{1}{m^2}$ as the third term, etc., it is evident that the first term takes the form given.

his value is the reciprocal of the Naperian Base e or we may write

$$\lim_{m \rightarrow \infty} \left(1 - \frac{1}{m}\right)^m = \frac{1}{e}$$

Substituting in formula (II), this gives:

$$Q = \left(\frac{1}{e}\right)^x = e^{-x} \quad (\text{III})$$

Whence $\log Q = -x \cdot \log e$, or using the base e

$$-x = \log_e Q; = \log_e (1 - P) \quad (\text{IV})$$

The significance of the minus sign is simply that since Q is a proper fraction, its logarithm is negative, thereby cancelling the minus sign before the x .

Since x is the number of *B. coli* in the sample tested, by using 1 cc. samples in our computations, we can obtain the *B. coli* factors for various percentages (P) of positive tests, so that it will only be necessary to divide the proper factor by the size of the sample used to obtain the number of *B. coli* per cubic centimeter. These factors have been computed and plotted in figure 1.

EXPECTED ERRORS¹

The results obtained by the method heretofore developed are subject to errors of sampling usual to values based upon obser-

¹ These formulae should not be confused with the usual formulae for probable error — $r = 0.6745 \sqrt{\frac{\sum r^2}{n-1}}$ and allied forms used in the method of least squares for the adjustment of observations. Both formulae are developed from the same basic conception of probability and fundamental probability series. However, the least square formulae assume that a fixed value, such as an angle, the distance between two points, etc., is to be ascertained, and that the errors are known to be small, as in the case of a transit for instance, they are known to be less than the smallest graduation on the plate.

The formulae here given are better adapted to statistical work, where larger and more irregular variations are to be expected, and indeed the quantity sought itself often undergoes changes during the investigation. They make use of mathematical conceptions of expectation, and approach more closely to an evaluation of the laws of chance than do the older and more familiar formula.

vational data. If the colon bacilli could be picked out and enumerated with certainty in the sample selected for testing, the result would be subject to error due to chance selection of the sample, this error being the same that must be reckoned with in the determination of morbidity and mortality rates, wherein the distribution of positive and negative cases are governed by laws of chance beyond control of the investigator; but their actual number can be determined by direct count. Here, however, the number of *B. coli* is estimated from the proportion of positive tests, involving an additional source of error, in the determination of this proportion. To determine P accurately requires a large number of tests, in order that all the possible combinations of *B. coli* and water droplets (as determined by the various terms of $(p + q)^s$) may have a fair chance to influence the result in proportion to their probability of existence.

Assume each cubic centimeter to be composed of s minute particles of which a are colon bacilli and B are droplets of water, other bacteria and matter; so that $a + B = s$. Then the probability that any one particle withdrawn in the sample for analysis is a colon bacillus is:

$$p_c = \frac{a_c}{s}$$

and by the same reasoning as before

$$q_c = 1 - p_c$$

Now it can be shown that the most probable or expected number of *B. coli* in a sample of s particles, or

$$e(a_c) = sp_c$$

and further, that the *expected error* of this expected value is:

$$\epsilon(a_c) = \sqrt{spq}$$

If instead of the 1 cc. sample of s particles, sets of N samples of n cc.'s each had been used, the total number of particles would have been Nns and the number of coli Nna .

$$\epsilon(Nna) = Nn \cdot \epsilon(a) = \sqrt{Nns pq}$$

$$\epsilon(a_N) = \sqrt{\frac{spq}{Nn}} = \sqrt{\frac{s}{Nn} \cdot \frac{a}{s} \cdot q}$$

Wherein the s 's cancel and q approaches 1 as s is indefinitely increased, giving as the final form

$$\epsilon(a_N) = \sqrt{\frac{a}{Nn}} \quad (\text{VIII})$$

This formula gives the expected error in the number of *B. coli* per cubic centimeter on the assumption that a is the actual existing number per cubic centimeter (since in developing the formula $\frac{a}{s}$ was assumed to be equal to p , the theoretical probability.) Since in actual practice a is not exactly known and cannot be exactly determined, the value as determined from the set of tests is used, or preferably the average value of a series of sets. It further assumes that a can be determined by direct count. In the present instance however, a is determined indirectly, being a function of Q , and its value is subject to the further error in determining Q . In formula VII, dividing through by s , we have:

$$\epsilon\left(\frac{a}{s}\right) = \epsilon(p) = \sqrt{\frac{pq}{s}} \quad (\text{IX})$$

Substituting P , Q and N gives

$$\epsilon(Q) = \sqrt{\frac{PQ}{N}} \quad (\text{X})$$

Whence a as a function of Q is

$$a = \frac{1}{n} \text{Log}_e \left(Q - \sqrt{\frac{PQ}{N}} \right)$$

Substituting in VIII gives

$$\epsilon(a_N) = \pm \sqrt{\frac{\log_e \left(Q - \sqrt{\frac{PQ}{N}} \right)}{N n^2}} \quad (\text{XI})$$

which is the formula for the expected error in the number of *B. coli* per cubic centimeter as computed from the value of Q found in a single set of N tests of n cc's each (n being a whole number or fraction). It assumes Q to be correctly known. As this is impossible the next best thing is to take Q as the average of a number of sets of tests.

From formula XI the errors for $N = 30, 100$ and 360 corresponding to series of tests of one month's, one hundred days' and one year's duration, have been computed and plotted in figure 1, assuming one cubic centimeter samples, or $n = 1$. For other values of n the error, as given must be divided by n to obtain the proper value. In this way the range in values corresponding to different proportions of positive tests is visualized.

The frequency with which errors larger than the expected error may occur is given by the formula:

$$T = 1 - \frac{1}{\lambda^2} \quad (\text{XII})$$

where λ is the ratio of the error in question to the expected error. Thus the probability of an error not over twice the expected is

$$T_2 = 1 - \left(\frac{1}{2}\right)^2 = 0.75$$

or in other words, there is one chance in four that the error, will be over twice the expected error. In figure 1, curves have been plotted giving the errors which may be expected once in twelve monthly sets and once in four yearly sets.

LIMITATIONS OF THE *B. COLI* FACTOR THEORY

As is the case with all formulae, whether of theoretical or empirical derivation, the theory herein developed is subject to

certain limitations in its practical application. These limitations have been studied by the aid of the error equations and the following results have been reached.

1. The proportion of positive tests (P) should be based on at least 10 tests; if less are used, the results may be entirely unreasonable due to large errors.

2. The accuracy of results for a given number of tests increases as the value of P increases, so that small values especially below $P = 0.30$ should be avoided if possible.

3. Above about 0.85 the curve, becomes so flat that a small variation in P causes a large variation in the *B. coli* count thus imposing an upper limit.

4. From several points of view a value of $P = 0.65$ gives the closest results for a given number of tests, so that as far as practical, the size of samples should be selected to give approximately this proportion of positive tests.

APPLICATION OF THE B. COLI FACTOR THEORY

In recognition of the frequent mathematical short-comings of bacteriologists, figure 1 has been prepared. By its use it is only necessary to determine the proportion of positive tests in a set, obtain the corresponding factor, from figure 1, and divide this by the size of sample, in order to obtain the *B. coli* per cubic centimeter. The range of variation for the number of samples most commonly used is also shown.

Example:

Data:

Number of tests made, 31.

Number of positive results, 13.

Size of sample, 0.10 cc.

Computations:

$P = 13 \div 31 = 0.42.$

Coli factor corresponding to 0.42 = 0.55.

$0.55 \div 0.10 = 5.5$ coli per cubic centimeter.

Expected error:

Variation for $P = 0.42$ and $N = 30$ (from figure 1) = from 0.43 to 0.68.

Dividing by 0.10 ($= n$), variation in count = from 4.3 to 6.8.

$(6.8 \text{ minus } 4.3) \div 2 = \pm 1.25 = \text{Expected error.}$

IRREGULAR GROUPING OF POSITIVE TESTS

Every attempt to express the degree of purity of a water supply in terms of the number of colon bacilli is beset with the difficulty that the number of such bacilli varies from day to day. The foregoing theory has assumed that this number is constant while the series of tests was being made, which would place the series in the group known as "normal" mathematically. Actually the *B. coli* content varies in a more or less erratic manner from test to test. This forms a serious obstacle in any attempt to establish a standard of purity, since a series in which all the positive results occur in one group would give the same average as another in which the positive results were evenly scattered, provided the number of positive tests were the same in both cases. By treating a series of tests as if it were a normal series, and then comparing the results with those for a similar number of total and positive tests as given in the foregoing series it is possible to obtain an index of the disturbancy of the series under investigation.

It is usual in a series of observational data to determine the mean, which is the most probable value. By subtracting each observation from the mean the residuals are found which are then squared and summed up. Dividing this summation by the number of observations and extracting the square root gives the *mean error* usually indicated by δ . These operations can be represented by the formula

$$\delta = \sqrt{\frac{(a_1 - M)^2 + (a_2 - M)^2 + \dots + (a_n - M)^2}{n}} \quad (\text{XII})$$

δ is the mean error in a as determined by a single set of tests. a_1, a_2, a_3 , etc., are the values as determined in the individual

sets of tests. M is the mean or average value of a as determined from ν sets of tests. Under ideal conditions (such as were assumed prior to this section of the paper) δ is equal to ϵ of formula XI.

The mean error in a single set (δ) should not be confused with the error of the mean (M) which is

$$\epsilon(M) = \frac{\delta}{\sqrt{\nu}} \quad (\text{XIV})$$

Of course if a large number of tests (say a year's) is considered as a single set then δ cannot be computed and the expected error will be found by the formula for $\epsilon(a)$ and will correspond to $\epsilon(M)$.

The relationship between δ and ϵ for a series of ν sets gives us two criteria by which to measure the disturbancy or departure from the ideal conditions of uniform distribution of *B. coli* in the water throughout the tests, assumed in the discussion of a normal series.

The first of these is the Lexian ration L , defined as

$$L = \frac{\delta}{\epsilon} \quad (\text{XV})$$

The second of these is the Charlier coefficient of disturbancy

$$100 p = 100 \sqrt{\frac{\delta^2 - \epsilon^2}{M}} * \quad (\text{XVI})$$

which has the advantage over L of not being affected by variations in the size of the samples.

It will be seen that for normal series $L = 1$ and $100 p = 0$.

The application of these criteria to the *B. coli* test is best shown by an example:

Data:

Size of samples, 10 cc.

Number of sets, 36.

Number of tests, 360.

Number of positive tests, 32.

Distribution of tests, given in tabulation p. 260.

* The Charlier coefficient is really a measure of the deviation in excess of the expected error for a normal series.

I. Treating these tests as a normal series which assumes that the coli content does not vary during the tests and that the results are only subject to the usual errors of sampling:

a. Proportion of positive tests

$$P = 32 \div 360 = 0.089$$

$$Q = 1.00 - 0.089 = 0.911$$

b. *B. coli* per cubic centimeter:

$$P = 0.089; \text{Factor (from figure 1)} = 0.089; \times \frac{1}{10} = 0.0089 \\ \text{coli per cubic centimeter}$$

c. Expected error in *B. coli* per cubic centimeter:

$$\epsilon(a_N) = \sqrt{\frac{\log \left(0.911 - \sqrt{\frac{0.089 \times 0.911}{360}} \right)}{360 \times 100}} = 0.0018 \text{ per cubic}$$

centimeter = ± 20 per cent

II. To take account of the irregular distribution of positive tests first divide the daily tests into sets of ten each; then determine the proportion of positive tests — P — for each set, by dividing the number of positive tests in the set by 10. Find the *B. coli* factor and *B. coli* per cubic centimeter for each set from its value of P . Then find the average coli per cubic centimeter from the 36 sets. By subtracting the value for each set from this average the deviations are found, and from these the mean error. (See p. 260)

Mean error in a single set (δ) = $\sqrt{\frac{0.038221}{36}} = \pm 0.032 = \pm 248$
per cent

Expected error in a single set (ϵ)* = $\sqrt{\frac{.013}{10 \times 10}} = \pm 0.011 = \pm 85$
per cent

$$L = \frac{0.032}{0.011} = 2.9 = \text{Lexian ratio.}$$

$$100 p = 100 \sqrt{\frac{0.032^2 - 0.011^2}{0.013}} = 73 = \text{Charlier coefficient}$$

*Using the formulae $e(a) = \sqrt{\frac{a}{Nn}}$, where $N = 10$ and $n = 10$.

Mean error of the mean $(0.013) = \frac{0.032}{\sqrt{36}} = \pm 0.005 = \pm 38$ per cent

Thus it is seen that by taking the average for the year (or considering the *B. coli* in the water to be constant) a value of 0.0089 ± 0.0018 was obtained. By arranging the data in a

TABLE 1

The Lexian ratio as a measure of the disturbancy of a year's tests when considered in groups of ten

Number of tests 360
Number of tests in each set 10

LEXIAN RATIO	RATIO OF MAXIMUM B. COLI IN ANY ONE SET TO THE AVERAGE FOR THE YEAR (ASSUMING UNIFORM DISTRIBUTION)					
	P 0.1	P 0.2	P 0.3	P 0.4	P 0.5	P 0.6
0	1.0	1.0	1.0	1.0	1.0	1.0
0.5	1.2	1.1	1.0	1.0	1.0	1.0
1.0*	2.0	1.5	1.25	1.1	1.0	1.0
1.5	3.5	2.1	1.8	1.5	0.3	1.2
2.0	5.3	3.0	2.4	2.0	1.8	1.6
2.5	8.0	4.0	3.2	2.8	2.4	2.0†
3.0	11.0	5.5	4.0	3.5	3.1†	2.7
3.5	14.5	7.3	5.0	4.5†	4.0	3.5
4.0	18.0	9.0	6.2†	5.4	4.9	4.3
4.5	23.0†	11.5†	7.5	6.3	5.9	5.0

Values of Lexian ratio which will allow a variation from the average for the year as indicated

P	1.5:1	2.0:1	2.5:1	3.0:1
0.1	0.75	1.00	1.20	1.30
0.2	1.00	1.40	1.75	2.00
0.3	1.25	1.75	2.05	2.40
0.4	1.50	2.00	2.30	2.70
0.5	1.75	2.20	2.60	2.90
0.6	1.95	2.50	2.80	3.20

*This line gives the normal error distribution.

†These values correspond to $P = 0.9$ in all the sets containing positive results and therefore represent the above ratio for the most unbalanced grouping possible with sets of ten tests each.

Distribution in daily tests for one year

SET	P	B. COLI FACTOR	B. COLI PER CUBIC CENTIMETER	DEVIATION	(DEVIATION) ²
1	0.0	0.0	0.0	-0.013	0.000169
2	0.1	0.1	0.01	-0.003	0.000009
3	0.0	0.0	0.0	-0.013	0.000169
4	0.1	0.1	0.01	-0.003	0.000009
5	0.2	0.22	0.22	0.009	0.000081
6	0.1	0.1	0.01	-0.003	0.000009
7	0.0	0.0	0.0	-0.013	0.000169
8	0.0	0.0	0.0	-0.003	0.000009
9	0.4	0.5	0.05	0.037	0.001370
10	0.0	0.0	0.0	-0.013	0.000169
11	0.1	0.1	0.01	-0.003	0.000009
12	0.1	0.1	0.01	-0.003	0.000009
13	0.0	0.0	0.0	-0.013	0.000169
14	0.0	0.0	0.0	-0.013	0.000169
15	0.0	0.0	0.0	-0.013	0.000169
16	0.0	0.0	0.0	-0.013	0.000169
17	0.7	1.2	0.12	0.107	0.011450
18	0.8	1.6	0.16	0.147	0.021600
19	0.2	0.22	0.22	0.009	0.000081
20	0.0	0.0	0.0	-0.013	0.000169
21	0.0	0.0	0.0	-0.013	0.000169
22	0.0	0.0	0.0	-0.013	0.000169
23	0.0	0.0	0.0	-0.013	0.000169
24	0.1	0.1	0.01	-0.003	0.000009
25	0.0	0.0	0.0	-0.013	0.000169
26	0.0	0.0	0.0	-0.013	0.000169
27	0.0	0.0	0.0	-0.013	0.000169
28	0.0	0.0	0.0	-0.013	0.000169
29	0.1	0.1	0.1	-0.003	0.000009
30	0.0	0.0	0.0	-0.013	0.000169
31	0.0	0.0	0.0	-0.013	0.000169
32	0.1	0.1	0.01	-0.003	0.000009
33	0.0	0.0	0.0	-0.013	0.000169
34	0.0	0.0	0.0	-0.013	0.000169
35	0.1	0.1	0.1	-0.003	0.000009
36	0.0	0.0	0.0	-0.013	0.000169
Mean.....		0.13	0.013		0.038221

series of sets so as to take account of fluctuations a value of 0.013 ± 0.005 resulted, or 1.7 times as large. The values of L and $100 p$ show a large disturbancy.

In checking the distribution by this method the number of tests assumed as one set has some effect on the results. In general the number of tests in a set should be the reciprocal of the proportion of positive tests to the total tests for the year, provided however, that the number of tests per set should not be less than ten. The proportion of positive tests in any set should not exceed 0.85, owing to the uncertainty of the *B. coli* factor for larger values. If a large number of consecutive positive tests occur, the series cannot be studied in this manner, since the results are then indeterminate. Such a condition would rather reflect on the analyst's judgment in selecting the size of samples, if he is following the methods given in this paper. It should be noted that it is not necessary to use the same size of sample throughout the year since each set of ten is treated independently. The size should be varied from set to set as required to maintain values as near the optimum as possible.

A careful investigation of these criteria leads the writer to believe that the Lexian ratio is the more useful of the two in studying the *B. coli* test. When L is equal to 1.0 the size of the errors and their distribution is such as may reasonably be expected in sampling a liquor of constant composition. When L is equal to 0.0 the distribution of results is absolutely uniform. Therefore, series in which L is fractional or unity, with perhaps an upper limit of 1.1 or 1.2 may be used with confidence in deriving averages, etc. For the higher values the inequality of distribution and size of errors increases very rapidly (about as L^2) and such series should be discarded for statistical work.

Table 1 gives some results based upon studies similar to the example just considered and will assist in giving a better understanding of the Lexian ratio.

THE "B. COLI CHARACTERISTIC"

The method of working up a year's results so as to find the mean, expected error and standard deviation has just been explained. It will now be shown how these data can be com-

bined so as to give by one figure a rational expression of the *B. coli* content, involving all these factors. Since the proposed form of expression gives the complete *B. coli* condition of the water for the period covered by the tests, it may safely be used in setting standards for purity, etc.

The basic idea is that the information most desired from the results of a series of *B. coli* tests is not an average value, but the maximum *B. coli* which are to be expected in any one test of the series, since it is this maximum value, which must be reckoned with as a factor in considering the possibility of transmission of the water-borne diseases. A water may show very low results for 364 days, but if it contains a large number of *B. coli* on one day of the year, it would be classed as dangerous although the average *B. coli* content would seem quite satisfactory. The actual maximum value obtained in any one test is not necessarily the maximum value to be expected and it is the latter in which we are interested.

By methods similar to those used in developing the expected error, it may be shown that the dispersion in a Lexian series is:

$$\delta_L^2 = \epsilon^2 + \frac{n^2 s^2 - ns}{N} \cdot \Sigma \cdot (P_v - P_o)^2$$

Where P_v is the probability in any set, and varies from set to set, and P_o is the average of all the various P_v 's. Substituting for P_v and P_o the corresponding expression $\frac{a_v}{s}$ and $\frac{a_o}{s}$ we obtain

$$\delta_L^2 = \epsilon^2 + \frac{n^2 s^2 - sn}{N} \Sigma \left(\frac{a_v}{ns} - \frac{a_o}{ns} \right)^2 \text{ or}$$

$$\delta_L^2 = \epsilon^2 + \frac{n^2 s^2 - ns}{n^2 s^2} \times \frac{\Sigma (a_v - a_o)^2}{N}$$

s , being the number of particles in a cubic centimeter, is very large, so that the factor

$$\frac{n^2 s^2 - ns}{n^2 s^2} \text{ or } 1 - \frac{1}{ns}$$

may be considered equal to unity. The last term is then the expression for the *standard deviation*, or

$$\delta_L = \epsilon^2 + \sigma^2$$

Where δ_L may be considered as the total expected error in any one set of those, comprising the series.

Assuming a year's tests to be composed of 36 sets of ten, the error to be expected once in 36 times is found by the criterion of Tchebycheff:

$$1 - \left(\frac{1}{\lambda}\right)^2 = \frac{35}{36}, \text{ whence } \lambda = 6$$

so that one set of ten may be expected to have a deviation of $6 \delta_L$.

Since the expected error is proportional to the square root of the number of tests, that for a single test in a set of ten would be the error in a set multiplied by $\sqrt{10}$ or 3.16. This gives as the final result

$$\delta_L = 3.16 \times 6 \times \sqrt{\epsilon^2 + \delta^2} = 18.96 \sqrt{\epsilon^2 + \delta^2}$$

and adding this to the average value for the coli, gives

$$C \frac{360}{10} = a_{10} + 18.96 \sqrt{e (a_{10})^2 + \delta_{a_{10}}^2}$$

$C \frac{360}{10}$ is the maximum number of *B. coli* per cubic centimeter to be expected once in 360 tests, these tests being considered in sets of ten. It is also a very concise and rational manner of stating the *B. coli* data relative to a water, since it combines in a logical and correctly weighted form, the average value, expected error and the standard deviation due to fluctuations in the water from test to test. For these reasons it may be called the *B. coli* characteristic. It is easily applied, since a_{10} , $e (a_{10})$ and $\delta_{a_{10}}$ are readily obtained by the methods given in previous

sections of this paper, and their combination involves only a simple computation. Applying this to the previous example, where:

$$a_{10} = 0.013$$

$$e(a_{10}) = 0.011$$

$$\delta(a_{10}) = 0.032$$

gives

$$C \frac{360}{10} = 0.013 + 18.96 \sqrt{0.011^2 + 0.032^2} = 0.668$$

In its general form, this formula is

$$\frac{C_{N'}}{N} = a_N + \sqrt{N'} (e(a_N)^2 + \delta a_N^2)$$

the symbols having the usual meaning.

Commenting on the foregoing method of interpreting a year's tests, the first question naturally is whether ten tests are sufficient to serve in computing the percentage of positive tests. It is a rather small number for such a purpose, but even so, the ten day period embraced in each set of ten tests, is already longer than desirable to allow the daily variations to assert themselves in the average. Therefore, if this method is to be accommodated to the routine practice of one test daily, the ten day period is probably as good as can be chosen. We must then expect an error of from 10 per cent for 0.9 positive to 100 per cent for 0.1 positive and averaging probably 25 per cent; this error being added into the *B. coli* characteristic and thus being taken into account. Selection of a size of sample to give positive results in the neighborhood of 0.65 will assist greatly in securing reliable results, and if more accurate data are desired, a series of ten fermentation tubes can be run every day, since with proper arrangements this would require little additional time.

In deriving a *B. coli* value from a series of tests, the assumption is that the *B. coli* content remains constant during the series, which is not actually the case. The factors causing change are

numerous and include: river stages, wind velocity and direction, temperature conditions, season of year, time of day, etc. Probably the various combinations of these conditions are very numerous. On the other hand, it must be recognized that there are equalizing tendencies due to the time element and to dilution. Such damping effects play a much more important part in the case of tests on water supplies drawn from rivers and lakes, than in the case of tests on crude sewage. If a sufficiently large number of tests are taken the variation from set to set as given by the standard deviation should cover as well the range in values to be expected from test to test. The 36 sets obtained in a year's tests are probably insufficient but must be used as the best data available.

It should be realized that even with 360 tests this method gives results that are far from exact, but they are along rational lines and can be increased in accuracy by increasing the number of tests.

SIGNIFICANCE OF REPEATED POSITIVE RESULTS

Considerable significance attaches to repeated positive tests when using a size of sample which gives a low percentage of positive tests. If 10 per cent of the tests are positive or $P = 0.1$, then the probability of obtaining two consecutive positive tests would be $\frac{1}{10} \times \frac{1}{10}$ or 0.01: and for three consecutive positive tests, $\frac{1}{10} \times \frac{1}{10} \times \frac{1}{10}$ or 0.001. In general if a percentage of positive tests, P , has been established by a series of tests on samples of a certain size, the probability of obtaining two consecutive positive tests is P^2 and of three such tests P^3 , etc. This affords a ready means for detecting sudden increases in the number of *B. coli* or, in investigating old records, assists in identifying periods of more than the average pollution.

THE USE OF THE NITRATE-REDUCTION TEST IN CHARACTERIZING BACTERIA

H. J. CONN AND R. S. BREED

From the New York Agricultural Experiment Station, Geneva, New York

Received for publication, January 29, 1919

INTRODUCTION

For some time bacteriologists have used the ability to reduce nitrates as a criterion for distinguishing certain kinds of bacteria. The test has been regarded as of so much diagnostic value that it has been included in nearly all schemes of bacterial characterization, among the most important of which is the descriptive chart adopted by the Society of American Bacteriologists. An investigation of this test was begun as a result of some student work done under the direction of one of us (B) upon certain cultures of the colon group. Such very irregular results were obtained in regard to the power of reducing nitrates that the method of making the test was decided to be unsatisfactory. Further work was therefore undertaken, at first by students and volunteer assistants, and then by one of us (C) as a contribution to the work of the Committee on the Descriptive Chart appointed by the Society of American Bacteriologists. For the earlier part of the work much credit is due to Emma Edson Breed, H. M. Weeter, and H. V. Grant.

TECHNIC

1. *Formula of medium.* An attempt was made at first to follow the "standard" technic adopted by the American Public Health Association (1905, 1912). It was soon found, however, that a standard technic was sadly lacking. In the 1905 Report of the Committee on Standard Methods of Water Analysis the following directions are given:

Dissolve 1 gram peptone in 1 liter of tap water, and add 2 grams of nitrite-free potassium nitrate. It is convenient to prepare a stock solution of potassium nitrate by dissolving 4 grams of solid nitrate in 100 cc. of distilled water and use 5 cc. of this solution in the above formula.

A little arithmetic will show that to follow the direction given in the first sentence would give a 0.2 per cent solution, while that in the second sentence would give a 0.02 per cent solution of the nitrate. In the 1912 report an attempt was evidently made to correct this disagreement, because the two grams in the first sentence was changed to 0.2 gram; but by some slip the 4 grams in the second sentence was also changed to 0.4 gram, thus giving a 0.002 per cent solution. With this inconsistency there has naturally been a great variation in the "standard" nitrate broths used by different bacteriologists. This is shown by a survey of the literature. Thus Gorham (1901) recommends 1 gram of peptone and 0.2 gram of potassium nitrate (i.e., 0.02 per cent); while Chester (1901) recommends a formula different from any of the above—10 grams of peptone and 0.02 gram (i.e., 0.002 per cent) of *sodium* nitrate. Apparently in preparing a formula for nitrate broth the figures 1 for the peptone and 2 for the nitrate have been considered more important than the position of the decimal point in either case. This peculiar irregularity has already been mentioned by one of us (Breed, 1915).

In the early part of this work two different nitrate broths were used independently by two of the workers, each thinking he was following the standard technic; both contained 0.1 per cent peptone but one contained 0.2 per cent KNO_3 and the other 0.02 per cent KNO_3 . When the discrepancy was noticed, the two media were compared, as were other different nitrate media, all of them within the limits of variation of the formulae given above. Later, other arbitrary variations were made for special purposes, as will be explained later.

2. *The nitrite test.* Two different variations of the official nitrite test were used in the first part of this work, differing only in the quantity of the reagents used. Each variation was sup-

posed to be "standard" by the particular investigator employing it, owing to two different interpretations of the standard methods. On page 120 of the report for 1912 the following technic is given: "Remove 3 cc. of the culture to a clean test tube and add 2 cc. of each of the naphthylamine solution and the sulphanilic acid solutions described under the determination of nitrites (see p. 22)." Three of the workers followed these directions exactly. Another, working independently, looked up page 22 and found that at this place the directions were to add 2 cc. to 100 cc. of the water to be tested, and concluded that the proportions of reagents given on page 120 must have been a mistake; so he used the reagents in the proportion of about 2 parts to 100 parts of the medium, i.e., a few drops to the culture tube. Later these two methods of using the reagents were compared, and the latter method was found to be the better. Using larger quantities of the reagent was found to dilute the nitrite present to such an extent as to obscure the reaction in cases where only small amounts of nitrite had been formed, especially when the reagents were old and somewhat discolored. As a result, in the latter part of the work, only a few drops of each reagent were added to the culture.

A still different test was used in some of the early part of the work: the potassium-iodide-starch test, as described by Erwin Smith (1905). This test compared very favorably with the official test, but proved a little less delicate and the reagents were found to deteriorate more rapidly; so its use was discontinued. This test is preferred by Harding (1910) just because it is less delicate; but as the present work progressed, the need of a delicate nitrite test was emphasized more and more clearly.

WORK WITH THE COLON GROUP

In 1912, one of the students taking part in the work (E. E. B.) observed considerable irregularity in the nitrate-reduction test in the case of fifty different cultures of the colon group isolated from polluted water. These cultures included all four of the commonly recognized types, as distinguished by their fermen-

tative reactions with sucrose and dulcitol. They were tested in triplicate in 0.02 per cent nitrate broth (with 0.1 per cent peptone), some giving negative results in all three tubes, others giving inconsistent results, while the majority of the cultures gave consistent positive results as was expected. In 1913 the cultures giving no positive reactions the first time were tested again by the same student, using the same medium, and all but three of the cultures were found to produce nitrite in at least one out of three tubes. These three cultures and one other (which had shown nitrite in only one tube each time) were tested a third time with consistently positive results. These findings are listed in table 1 under the heading "Series of 1912-13." In all, about 64 per cent of the tests were positive, counting each tube in a set of triplicates as a single test. The fact that every culture gave a positive reaction in the end suggested that all might be nitrate-reducers, but that the methods of testing were such that they did not develop this ability in more than about two-thirds of the cases.

Later in 1913 (see table 1, Series of 1913) the same cultures were tested in triplicate again by another student (H. M. W.) using the same medium and the same methods generally, recording the results similarly. By this student 37 cultures were tested twice, 15 of them three times. Until the formula of the medium was varied, only about 37 per cent of the tests were positive. In regard to the individual cultures, there was practically no agreement with the results of the first student: only three cultures (nos. 11, 14 and 35) gave consistently positive reactions on both occasions.

In the course of this work it was noticed that no strain grew well in the medium used; so some preliminary work was done in varying the composition of the medium (last column under "Series of 1913," table 1). Twelve cultures that had given negative reactions in the majority of cases were tested again in a medium containing 0.2 per cent (instead of 0.1 per cent) peptone. The growth in this medium was noticeably better, and a distinct nitrite reaction was obtained in all 36 tubes.

TABLE 1

Nitrate reduction by organisms of the colon group in 0.02 per cent nitrate broth. Tests made in triplicate; + and - indicate presence and absence respectively of a distinct nitrite reaction in all three tubes; ± indicates a distinct nitrite reaction in two out of the three tubes, † in only one of the three tubes. T indicates mere trace of nitrite.

CULTURE NUMBER	SERIES OF 1912-13			SERIES OF 1913				SERIES OF 1914		
	Peptone 0.1%			Peptone 0.1%			Peptone 0.2%*	Peptone 0.1%	Peptone 0.2%	Peptone 0.5%
	First test	Second test	Third test	First test	Second test	Third test				
1	-	+		-	-	+				
2	-	†		-	†			†	+	+
3	+			-	+			†	+	+
4	±	+		-	±			-	+	+
5	-	+		-	+†			±	+	+
6	±	±		-	T	+		-	+	+
7	+			-	-	+	+	+	+	+
8	-	+		±				-	+	+
9	+			-	T†			-	+	+
10	-			-	†			±	+	+
11	+			+	+			+	+	+
12	±			-	-	+		†	+	+
13	+			-	±			+	+	+
14	+			T	+					
15	+			±				+	+	+
16	T?	+		-	T	-	+	+	+	+
17	-	+		-	+					
18	±			±				-	+	+
19	+			-	±†			-	+	+
20	-	+		-	†	-	+	-	+	+
21	±	+		+				-	+	+
22	-	+		-	†			+	+	+
23	+			-	T	+		±	+	+
24	+			-	±			-	+	+
25	-	+		-	+†			+	+	+
26	-	+		-	-	+	+			
27	†	+		-	+			-	+	+
28	+			±				†	+	+
29	+			-	-†	+	+	†	+	+
30	+			-	†			±	±	+
31	-	+		-	-	±	+	-†	+	+
32	+			†	+			-	+	+
33	+			-				-	±†	+
34	-	+		-	+	+	+	±	+	+
35	+			+				+	+	+

TABLE 1—*Continued*

CULTURE NUMBER	SERIES OF 1912-13			SERIES OF 1913				SERIES OF 1914		
	Peptone 0.1%			Peptone 0.1%			Peptone 0.2%*	Peptone 0.1%	Peptone 0.2%	Peptone 0.5%
	First test	Second test	Third test	First test	Second test	Third test				
36	+			—	+			+	+	+
37	+			—	—	+	+	±	+	+
38	—	+		—	—	—	+	±	+	+
39	+			—	—	+	+	—	+	+
40	±			—	—			—	+	+
41	—	—	+	+	±†			+	+	+
42				—	T	—	+	—	+	+
43	—	—	+	—	+			—	+	+
44	+			—	+	+	+	+	+	+
45	—	—	+	+	—	?	+	±	+	+
46	±	±	+	+				—	+	+
47	+			±	T			—	+	+
48	+			+	±			±	+	+

* This medium contained only 0.01 per cent nitrate.

† One of the tubes showed nitrite present in mere traces only.

‡ One of the tubes showed a distinct positive reaction.

The idea suggested by the last mentioned tests was followed up by a third student (H. V. G.) the next year. Parallel tests were made in nitrate broth containing 0.1, 0.2, and 0.5 per cent peptone respectively (Series of 1914, table 1). With 0.1 per cent peptone only 42 per cent of positive results were obtained, and the agreement of the individual cultures with their previous behavior was no greater than it had proved to be the preceding year. With 0.2 per cent peptone 98.5 per cent of the tests (129 out of 131 tubes) gave positive results, every culture showing a positive reaction in at least two of the tubes. With 0.5 per cent peptone all of the cultures gave positive reactions in all three of the triplicate tubes. Further tests not listed in the table were made with a medium containing 0.1 per cent peptone and 0.2 per cent (instead of 0.02) per cent nitrate. This use of ten times the original amount of nitrate was found to have no influence on the results, the irregularity proving as great as with 0.1 per cent peptone and 0.02 per cent nitrate.

The conclusion reached by this work was that inconsistent results of the nitrate-reduction test may be expected with organisms of the colon group unless enough peptone is present to furnish these bacteria with conditions favorable to their vigorous growth. With 0.1 per cent peptone the growth was generally poor; with 0.2 per cent fairly good; and with 0.5 per cent very good. The amount of nitrate present apparently had little influence on the results. The important matter was a vigorous growth of the organisms; and under conditions allowing vigorous growth all the cultures of the colon group tested proved to reduce nitrate to nitrite.

WORK WITH *BACILLUS CEREUS* FRANKLAND

As an organism to contrast with those of the colon group, *B. cereus* Frankland¹ was chosen. *B. cereus* grows well in the presence of considerable organic matter, but it does not seem to require the large amounts of nitrogenous material that the colon organisms do. The cultures of *B. cereus* used were isolated by one of us (C) from soil.

Ten out of 130 cultures, apparently all *B. cereus*, failed to produce nitrite when tested, promptly after isolation from soil, in broth containing 0.1 per cent peptone and 0.2 per cent KNO_3 . One of these ten cultures (which we will denote culture x) was tested again two years later (table 2, test 2) together with several typical *B. cereus* cultures (which we will call cultures A to G respectively) that had produced nitrite the first time. This work was done by a different investigator (H. V. G.) from the one who made the original tests. In order to see whether the explanation for the disagreement previously found might be due to the same cause as the disagreement in the case of the colon organisms, the four following nitrate broths were used:

- 0.1 per cent peptone, 0.2 per cent nitrate
- 0.1 per cent peptone, 0.02 per cent nitrate
- 0.2 per cent peptone, 0.02 per cent nitrate
- 0.5 per cent peptone, 0.02 per cent nitrate

¹ This organism was identified by means of the characteristics previously described (Conn 1917).

TABLE 2

Nitrite production by B. cereus in various nitrate media. Tests made in duplicate. Each + or - sign indicates presence or absence of nitrite in all three tubes of one set. The sign ± indicates disagreement among three parallel tubes

Medium containing KNO ₃ { Peptone	ORIGINAL 11-1161 1893			SECOND TEST, 1914			THIRD TEST, 1914			FOURTH TEST, 1914			FIFTH TEST, 1915						SIXTH TEST, 1916	SEVENTH TEST, 1918
	0.2% 0.1%	0.2% 0.1%	0.2% 0.1%	0.02% 0.1%	0.02% 0.2%	0.02% 0.5%	0.2% 0.1%	0.2% 0.5%	0.2% 0.5%	0.2% 0.1%	0.2% 0.5%	0.2% 0.1%	0.2% 0.5%	0.02% 0.1%	0.02% 0.2%	0.02% 0.5%	0.2% 0.1%	0.2% 0.5%	0.1% 0.2%	0.1% 0.2%
Culture A....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture B.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture C.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture D.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture E.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture F.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture G.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture H.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture X.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture Y.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture a.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Culture b.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Culture c.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Culture d.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Culture x.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* This medium contained 1 per cent glucose.

† Nitrate-peptone agar. Test made on agar slants.

‡ Five different nitrate agars used, containing no added organic matter except sugar. Tests made on agar slants. Each medium inoculated in triplicate and the three tubes tested on different days. Each + or - sign indicates presence or absence of nitrite in all fifteen tubes.

Each culture was inoculated in triplicate into each medium; and clear-cut positive nitrite reactions were obtained in all cases, even with culture x.

A few months later (test 3, table 2) cultures A and B, together with seven other typical nitrite-positive *B. cereus* cultures² were tested again in the first of the nitrate broths listed above, all giving such clear-cut positive reactions that no further investigation of these cultures were made. At the same time, six other cultures were retested in this same medium, and also in media of the following composition:

0.5 per cent peptone, 0.2 per cent nitrate;
0.5 per cent peptone, 1 per cent glucose; 0.2 per cent nitrate.

One of these six cultures (culture X, table 2) had given a positive reaction the first time, while the others (cultures a, b, c, d and x) had given negative reactions. The results showed no nitrite from cultures a, b, c, d, and none from culture X, while nitrite was found with culture x, as had been the case in test 2. In other words all the cultures except X and x showed agreement with the original test; but X had changed from nitrite-positive to nitrite-negative, x from nitrite-negative to nitrite-positive.

Test 4 was made a few weeks later. This test included cultures b, c, x and X, together with a fifth, which from its similarity to culture X will be denoted as culture Y. Cultures b, c, x and X gave the same results as in test 3. Culture Y, like culture X, had given a positive nitrite reaction at the time of isolation two years earlier, but now showed no production of nitrite.

No further tests of culture Y were made, but cultures X, a, b, c, d and x were tested again about a month later (test 5). This test was made in six different nitrate broths, the four used in test 2 together with two additional broths as follows:

0.2 per cent peptone, 0.2 per cent nitrate
0.5 per cent peptone, 0.2 per cent nitrate

² Not listed in Table 2, because no further work was done with these seven cultures.

All the cultures except culture c gave the same results as in the two preceding tests, no difference being observed between their behavior in the different media. Culture c gave distinctly negative results with the four media used in test 2; but with the two new media (that is, the media with 0.2 per cent nitrate combined with more than 0.1 per cent peptone) a discrepancy was observed on the tenth day, one of the two duplicate tubes of each medium tested giving a positive nitrite test.

Four years later culture a was tested again, still with negative results. In the meanwhile the advantage of agar for making the test in doubtful cases (see p. 277) had been learned, and so this culture at this time was tested also on beef-extract-peptone agar to which 0.1 per cent KNO_3 had been added. The results were still negative. Good growth, however, was obtained in all cases.

Test 7 was made a few months later. Three organisms were tested this time: H, a typical nitrite-positive *B. cereus* culture that had not been tested before since immediately after isolation five years before; culture X and culture b. These cultures were tested at this time in six different agar media, all but one of them containing no added organic matter except some sugar. Culture H gave positive results in all the media used; cultures X and b gave negative results throughout although one tube out of nine in the case of culture b gave a positive nitrite reaction on the seventh day (due probably to an impurity). One of the media used contained no possible source of ammonia except the nitrate, so a Nessler test was made to see if the nitrate had been converted into ammonia without accumulation of nitrite. This test also was negative.

As a result of this work it was concluded that with *B. cereus* the explanation of the irregularities is not as simple as in the case of the colon organisms. *B. cereus* grows well in almost any medium and ordinarily reduces nitrate to nitrite. Certain cultures, however, seem to lack this reducing power, either temporarily or permanently, although they grow well in the media used. In general, constantly negative or constantly positive results have been found with any particular culture. This sug-

gests that there may be two different species so closely related that they can be distinguished only by means of the nitrate test. Three cultures, however (X, Y, and x) and possibly a fourth (c) gave inconsistent results. Although this disagreement may possibly have been due to a contamination that ran out the original organism, the agreement in all other respects with the original descriptions makes this explanation doubtful. No other explanation of the irregularity has been found, however, unless it be assumed that cultures of *B. cereus* may lose or gain the power of reducing nitrates when cultivated in the laboratory.

WORK WITH FLUORESCENT PSEUDOMONADS

During the course of a study of soil bacteria, numerous cultures of fluorescent pseudomonads were obtained that appeared to be closely related although differing in certain particulars. One of the most noticeable points of difference was that some produced nitrite in nitrate broth containing 0.1 per cent peptone while others produced no nitrite under these conditions. With these organisms the growth was always fairly good; nevertheless it was felt that the difference might be due to causes similar to those affecting nitrate-reduction by the colon organisms. Upon testing the cultures in broth containing larger amounts of peptone (as much as 1 per cent) the same distinction between nitrate-producers and non-nitrite-producers held true; but in this broth a further difference appeared, some of the organisms producing gas (presumably free N), others producing no gas. In other words three groups of fluorescent pseudomonads were found upon inoculation into 1 per cent peptone solution containing nitrate: (1) producing nitrite and gas; (2) producing nitrite but no gas; (3) producing neither nitrite nor gas (in appreciable quantities).

As the fluorescent organisms grow rather better on the surface of agar than in a liquid medium, further work was carried on in a nitrate agar containing beef-extract 0.3 per cent, peptone 1 per cent, KNO_3 0.1 per cent. The nitrite test was made by pouring the reagents upon the surface of the agar slant after incubation;

gas was detected by means of bubbles and cracks in the medium. One day of incubation was enough to bring out the nitrite reaction with vigorous nitrate-reducers; but cultures were generally kept until the seventh day. This agar slant test was found to give the same results obtained with the 1 per cent peptone broth cultures; the same three groups were found as before.

TABLE 3

Composition of nitrate media used in tests listed in tables 4-7. Figures indicate grams per liter.

INGREDIENTS	MEDIA SYMBOLS*					
	P	DM	D	DL	S	DA
Agar†.....	15.0	15.0	15.0	15.0	15.0	15.0
Peptone.....	10.0					
Glucose.....		10.0	10.0	10.0		10.0
Lactose.....				5.0		
Sucrose.....					10.0	
KNO ₃	1.0	1.0	1.0	1.0	1.0	1.0
CaCl ₂		0.5	0.5	0.5	0.5	0.5
MgSO ₄		5.0				
K ₂ HPO ₄		5.0	0.5	0.5	0.5	0.5
NH ₄ Cl.....						2.0
Beef extract.....	3.0					

* In these media symbols, the letters represent the significant ingredients, as follows: P, peptone; D, glucose; M, magnesium sulphate; L, lactose; S, sucrose; A, ammonium chloride.

† "Bacto-agar" (a purified agar sold by the Digestive Ferments Co.) was used in all except Medium P.

For further testing six cultures were selected, two of each group. These six cultures will be denoted in this paper as follows:

- AA } Producing nitrite and gas in nitrate-peptone media.
- BB }
- A } Producing nitrite without gas in nitrate-peptone media.
- B }
- a } Producing neither nitrite nor gas in nitrate-peptone media.
- b }

These six cultures were retested not only on the nitrate-peptone agar previously used (test 1, table 4) but also on agar containing no nitrogen (disregarding impurities) except 0.1 per cent KNO_3 and with no added organic matter except sugar (tests 2-5, table 4). The object was to learn whether the nitrate was converted to ammonia. The reports of the Committee on Water Analysis (A. P. H. A. 1905, p. 150; 1912, p. 120) have called attention to the need of making the ammonia test, but have added that ammonia may also come from the peptone. Kligler (1913) emphasized the importance of this source of error. As the fluorescent organisms are all ammonifiers, it is impossible to tell whether the ammonia present in a nitrate-peptone medium comes from the nitrate or the peptone. Hence it was decided to grow them under conditions where there could be no source of ammonia except the nitrate, so that its presence would show reduction of the nitrate even though there was no accumulation of nitrite. The first media tried contained glucose (media DM, and DL, table 3) and were found to be unsatisfactory for the ammonia test because no glucose could be obtained free from ammonia. Lactose was found to have the same disadvantage, so finally sucrose (medium S, table 3) was employed as a source of energy.

Even before a satisfactory ammonia-free medium was obtained, some very interesting results were procured (see table 4). On these media all six cultures, even including strains a and b, were found to give the nitrite test, sometimes in mere traces, but often in appreciable quantities, as early as twenty-four hours after inoculation. The test on the ammonia-free medium S (test 4, table 4) gave the following results: Cultures AA and BB showed the presence of ammonia, as well as the nitrite and gas demonstrated on the peptone media; cultures A and B showed a very strong nitrite reaction, but a weak ammonia reaction after the fourth day; cultures a and b showed a moderate nitrite reaction and an ammonia reaction slightly stronger than with cultures A and B. The ammonia reactions were in no case strong enough to prove that the organisms were converting the nitrate rapidly into ammonia.

TABLE 4

Nitrate reduction by *Ps. fluorescens* in 0.1 per cent nitrate agar. First test.* Cultures inoculated in triplicate, but only one tube tested each day. Strength of reaction indicated as follows: +++ very strong, ++ strong, + distinct, T trace, - absent, ? doubtful.

	MEDIUM P.			MEDIUM DM.			MEDIUM DL.			MEDIUM S.				MEDIUM DA.		
	Nitrite present in			Nitrite present in			Nitrite present in			First day	Second day		Fourth day	Nitrite present in		
	1 day	4 days	7 days	1 day	4 days	7 days	1 day	2 days	3 days		Nitrite	Ammonia		1 day	2 days	3 days
Culture A.....	+++	T	-	++	++	+	++	++	++	?	++	-	++	++	++	++
Culture B.....	+++	+++	+	++	++	+	++	++	++	?	++	-	++	++	++	++
Culture a.....	-	-	T	+	+	T	+	+	+	?	+	+	+	-	-	-
Culture b.....	-	-	T	+	+	T	+	+	+	?	+	+	+	-	-	-
Culture AA.....	+	T†	+	++	++	+	++	++	++	?	++	+	+	+	+	+
Culture BB.....	+++	T†	-†	++	++	+	++	++	++	+	++	-	+	+	+	+

* In this test the five media were inoculated separately, on different days.

† Gas production shown by presence of cracks in the agar.

A further test (test 5, table 4) was then made on a synthetic agar (medium DA, table 3) containing nitrate, glucose, and ammonium chloride. Cultures A, B, a and b gave the same reactions (i.e., either nitrite-positive or nitrite-negative) as on peptone media; of the gas-producers, BB behaved as on the other media, but AA failed to produce gas. In other words, it was shown that cultures a and b were prevented from producing nitrite on the synthetic medium by the addition of ammonium chloride. This test was considered to show that some strains of fluorescent organisms, although capable of reducing nitrate, do not attack it if there is a more easily available source of nitrogen present.³ In this connection it is interesting to note that the nitrite reaction of these two organisms was stronger on the sucrose medium than on the media containing those sugars which had ammoniacal impurities.

The sixth test (table 5) was run a few months later. This test was a repetition of tests nos. 2 to 5, using the same four media together with one other glucose agar only slightly different (medium D, table 3). The same six cultures were tested, together with four others (laboratory cultures isolated from soil nearly six years previously): two gas-formers CC and DD, and two that were nitrite-negative in peptone media, c and d. Of these four cultures, DD was especially interesting. It had been kept under cultivation in the laboratory for the longest time of all, and when first tested on medium P (nitrate-peptone agar) six years after isolation, was found to be a weak gas-producer. During the next few months its power of gas-production apparently diminished until when the work recorded in table 5 was done it failed to produce gas on any medium. The possibility of a contamination being present and causing this discrepancy is not entirely excluded.

The four cultures AA, BB, A, B, a, and b, gave results agreeing fairly well with the earlier tests, except in regard to the gas-

³ Another possible explanation is that growth is so rapid in the presence of ammonium chloride that these organisms are able to use up the nitrite as fast as produced. This theory is not a satisfactory explanation, however, because the growth of Cultures a and b was better in the absence than in the presence of ammonium chloride in this medium.

TABLE 5

Nitrate reduction by P8. Fluorescens in 0.1 per cent nitrate agar. Second test. Cultures inoculated in triplicate, but only one tube tested each day.*
Strength of reaction indicated as follows: +++ very strong, ++ strong, + distinct, T trace, - absent, ? doubtful

CULTURE	MEDIUM D.				MEDIUM DL.				MEDIUM S.								MEDIUM DA.			
	Nitrite present in				Nitrite present in				2 days		4 days		5 days		7 days		2 days		4 days	
	2 days	4 days	5 days	7 days	2 days	4 days	5 days	7 days	Nitrite	Ammonia	Nitrite	Ammonia	Nitrite	Ammonia	Nitrite	Ammonia	Nitrite	Ammonia	2 days	4 days
A.....	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
B.....	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
a.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
b.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
c.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
d.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AA.....	++	+	+	+	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
BB.....	+++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
CC.....	+++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
DD.....	+	T	T	T	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

* In this test the five media were inoculated simultaneously.

† Gas production shown by presence of cracks in the agar.

production by AA and BB on certain media, a point which seems open to considerable variation. The two old cultures, c and d were found to be even less vigorous nitrate-reducers than a and b. Culture c produced no nitrite on any medium and only a trace of ammonia on medium S; culture d showed nitrite and ammonia on medium S, but no nitrite on any other medium. Culture c grew very poorly on medium S, a fact which undoubtedly explains its failure to reduce the nitrate on this synthetic medium.

It was therefore concluded that vigorous growth of the fluorescent organisms is as necessary as it is with the colon organisms, in order to get a satisfactory test for nitrate-reduction; but that a more common cause of error is that cultures do not always reduce the nitrate if a more readily available source of nitrogen is present. In the present work the difference between those cultures that could reduce nitrate under all conditions and those that could do so only in the absence of other sources of nitrogen remained constant; but, as the two sets of cultures resembled each other in all other respects and as various gradations were found between those that showed no nitrite in the presence of mere traces of ammonia and those that produced it even in the presence of peptone, it does not seem to be a question of two distinct species.

WORK WITH AN ORANGE CHROMOGENIC PSEUDOMONAS

Another organism chosen for study because of its irregularity in respect to the nitrite test was an orange chromogenic pseudomonas, a form very common in soil and water. It has recently been studied in this laboratory and a description of it is now in course of publication (Conn and Bright, 1919). It is concluded to be identical with *Bacillus caudatus* Wright (1895), although it has a polar flagellum. This organism grows so poorly in liquid media of all sorts that nitrate broth was realized from the beginning to be an unfair medium to use in the nitrate-reduction test, and the early irregular results were considered to be due to the poor growth in this broth. For that reason beef-

extract-peptone agar containing 0.1 per cent nitrate (medium P, table 3) was used in subsequent work.

A special study was first made of eight different strains of this organism, four of which strains had been separated into two or three substrains immediately after isolation from the soil, all of these substrains having been kept distinct during the laboratory cultivation. Including these separate substrains, 15 different cultures were studied. It was found (see test no. 1, table 6) that they could be divided into two groups, those producing abundant nitrite on nitrate-peptone agar and those showing no nitrite on this medium. The cultures may be listed as follows:

Group 1.—Producing nitrite

- A. A typical chromogenic strain.
- B. A typical chromogenic strain.
- $\left. \begin{array}{l} C_1 \\ C_2 \end{array} \right\}$ Two substrains of a typical chromogenic strain.
- $\left. \begin{array}{l} D_1 \\ D_2 \\ D_3 \end{array} \right\}$ Three substrains of a typical chromogenic strain.
- $\left. \begin{array}{l} X_1 \\ X_2 \\ X_3 \end{array} \right\}$ Three substrains of a non-chromogenic strain.

Group 2.—Not producing nitrite

- $\left. \begin{array}{l} a_1 \\ a_2 \\ a_3 \end{array} \right\}$ Three substrains of a chromogenic strain.
- b A chromogenic strain.
- c A chromogenic strain

On nitrate-peptone agar the agreement between the different substrains of any one original strain was complete. Some of the strains grew more vigorously than others. In general there was no correlation between vigor of growth and production of nitrite, although it was striking that after a few months cultivation in the laboratory all the nitrite-negative cultures died.

These fifteen cultures were tested upon synthetic nitrate media (tests 2-5, table 6), the same media used for the fluorescent organisms. Medium DM (test 2) gave rather surprising results, as nitrite was absent in all but two cases, and in one of these two cases was present in mere traces. This was not due to poor growth in all cases, for very good growth was obtained with cultures A and B and with all substrains of C and D; whereas culture X₂ grew poorly but gave the nitrite test. A later repetition of this test (see table 7) with A, B and all substrains of X showed nitrite to be produced by cultures A and B, but that the nitrite reaction with culture B disappeared after the first day. This suggests that the meaning of the negative reaction in test 2 (table 6) may have been in many cases that the cultures were examined after the nitrite had disappeared.

The poor growth of strains X, a, b, and c, noticed on this medium, was observed on all the other synthetic agars used.

This was undoubtedly due to the fact that these media contained no added organic matter except the sugars; for these four strains were found to be unable to attack any sugar.

The cultures were then tested on medium DL (test 3, table 6). A distinct nitrite reaction was obtained promptly with cultures A, B, C₁, C₂, D₁, D₂, and D₃ and on the seventh day with the non-chromogenic strain X₂. No trace of nitrite was observed with any of the cultures which had not produced nitrite in the earlier tests. The correlation between vigor of growth and strength of the nitrite reaction was quite marked. Test 4 was made upon medium S, containing sucrose instead of glucose and lactose, sucrose being used because of its freedom from ammoniacal impurities. Tests for both nitrite and ammonia were made. The results were very striking. Nitrite (at least in traces) was observed with every culture except one of the substrains (X₁) of the non-chromogenic strain. Appreciable amounts of ammonia were observed with every culture, showing nitrate-reduction to have occurred in all cases. As in the previous test, vigor of growth was distinctly correlated with the strength of the nitrite reaction, growth in this case tending to be rather better than on the lactose-glucose medium.

TABLE 7

Nitrate reduction by the orange chromogenic Pseudomonas in 0.1 per cent nitrate agar. Second test. Cultures inoculated in triplicate, but only one tube tested each day. Strength of reaction indicated as follows: +++ very strong, ++ strong, + distinct, T trace, - absent; ? doubtful.*

	MEDIUM P.				MEDIUM DM.				MEDIUM D.				MEDIUM DL.				MEDIUM S								MEDIUM DA.			
	Nitrite present in				Nitrite present in				Nitrite present in				Nitrite present in				First day		Second day		Fifth day		Seventh day		Nitrite present in			
	1 day	2 days	5 days	7 days	1 day	2 days	5 days	7 days	1 day	2 days	5 days	7 days	1 day	2 days	5 days	7 days	Nitrite	Ammonia	Nitrite	Ammonia	Nitrite	Ammonia	Nitrite	Ammonia	1 day	2 days	5 days	7 days
Culture A...	++				++	+			++	+			++	+			+		++	+			+	+	+	+	+	+
Culture B...	T		++		+		+		T		+		T		+		+		+	+	+	+	+	+	+	+	+	+
Culture E...	++	++	++		++	++	++		++	++	++		++	++	++		++	++	++	++	++	++	++	++	++	++	++	++
Culture F...	++	++	++		++	++	++		++	++	++		++	++	++		++	++	++	++	++	++	++	++	++	++	++	++
Culture G...	++	++	++		++	++	++		++	++	++		++	++	++		++	++	++	++	++	++	++	++	++	++	++	++
Culture X ₁ ...	-			++	+	+	+		T	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+
Culture X ₂ ...	-			+	+	+	+		+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+
Culture X ₃ ...	-			+	+	+	+		+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+
Culture X ₄ ...	-								+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+

*In this test the six media were inoculated simultaneously.

Later (test 5, table 6) a few of the cultures were tested upon agar (medium DA) containing ammonium chloride as well as nitrate and glucose. Nitrite was found with all seven of the strains that showed nitrite on nitrate-peptone agar (medium P), also with culture b, the one strain tested that had not shown nitrite on medium P, but not with the one substrain (X_2) of the non-chromogenic organism that was tested.

A few months later those cultures that were still alive were tested again (table 7), together with a few other strains of the same organism. This is a very difficult organism to keep living under laboratory conditions, and only cultures A, B, X_1 , X_2 , and X_3 were still alive. The strains which had failed to produce nitrite on the medium P had all died. The new cultures studied were:

E }
F } Three recent isolations from soil, all giving a strong nitrite
G } reaction on Medium P when first isolated.

X_4 A fourth substrain of the non-chromogenic strain, obtained by inoculating X_3 into sterile soil and reisolating after a few weeks.

These nine cultures were inoculated into the same media used in the earlier test and also into medium D.

The only essential difference noted from the results of the earlier tests was that the non-chromogenic cultures had now lost their power of producing nitrite upon any of the media tested, even including medium P. Cultures A, X_1 , X_2 , and X_4 still gave distinct ammonia reactions in medium S, and X_3 showed a trace of ammonia.

The conclusion drawn from these tests is that this pseudomonad is neither like the colon organisms nor like the fluorescent bacteria in respect to the nitrate-reduction test. Some available organic matter must be present in order to allow good growth; and yet it is possible for some strains to produce good growth without giving the nitrite test. There is evidence that in some cases, at least, this is because the nitrite is converted into ammonia as fast as produced, so that there is no accumulation of nitrite.

CONCLUSIONS

As a result of this work the conclusion was drawn that the nitrate-reduction test, as made by testing for nitrite in "standard" nitrate broth after a definite period of incubation, is not as simple as generally supposed. This test is open to several sources of error:

1. *Poor growth.* Any organism must be tested in some medium in which it makes good growth. If it grows poorly, the results are likely to be variable (as with the colon organisms in media containing less than 0.2 per cent peptone), and under such conditions absence of nitrite is of no significance. It is probably impossible to find any one medium in which all bacteria make satisfactory growth.

2. *Presence of more readily available nitrogen.* Some of the cultures studied (fluorescent pseudomonads) seem to be able to reduce nitrate only in the absence of ammoniacal (or amide) nitrogen. Their ability to reduce it does not show, therefore, on ordinary peptone media. Such behavior may sometimes be of diagnostic importance; but the fluorescent cultures studied which showed this characteristic differed in no other observed respect from typical *Ps. fluorescens* (vigorous nitrate-reducer) and are not thought to belong to a separate species.

3. *Reduction without accumulation of nitrite.* Some organisms (like certain strains of "*B. caudatus*") utilize the nitrite as fast or almost as fast as produced. It may thus be assimilated, converted into ammonia, or converted into free nitrogen. Free nitrogen can generally be detected by gas bubbles in the liquid or cracks in the agar. Ammonia can be detected only if the organism is growing in an ammonia-free medium containing no source of ammonia other than the nitrite; but many organisms are unable to grow under such conditions. Assimilation of the nitrite (either as nitrite or as ammonia) cannot be detected by any simple test.

In case of each of the four species (or groups of species) studied, a different explanation was found necessary to account for cultures showing no nitrite. Only in one case, that of *B. cereus*, did

investigation show the possibility of two species being concerned, one differing from the other in its ability to reduce nitrate. Inasmuch, therefore, as nitrate-reduction on any medium or under any condition whatsoever indicates an organism as a nitrate-reducer, the general conclusion of the present work is that no organism can be safely called a non-nitrate-reducer except as the result of exhaustive tests, too time-consuming to be made in routine bacteriological investigations.

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WATER SUPPLY FOR A SYRINGE BATH

RALPH R. MELLON

Department of Laboratories, Hahnemann Hospital, Rochester, New York

Received for publication, February 10, 1919

It is a matter of common laboratory knowledge that nickel plated syringes, steel needles, etc., should be boiled in an alkaline solution. Na_2CO_3 is often employed, although I prefer borax. Some workers have found it desirable to keep syringes in such solutions, in which case they are always ready for use. If tap water is employed, a deposit of lime salts may be deposited at the junction points of the syringes or in the needles, causing troubles of various kinds.

Distilled water will avert such difficulty, but it is necessary to conserve it, and if syringes are used often, it is desirable for obvious reasons, to have just enough water to cover them. I have devised a simple mechanism to supply such a bath with distilled water, almost automatically.

The bottle *A*, figure 1, rests on a shelf in a hood about 2 feet above the level of the bath *F*, which is (viewed from the inside in the figure). The outlet *b*, has a rubber tube connecting with a metal stop-cock inlet *c*, located in the side of the bath as near its bottom as possible. Another stop-cock the inner opening of which is located at *d*, indicates the desired level for the water. The bottle *A* has a two-holed rubber stopper with a piece of glass tubing in either hole. One leads to *d*, the other to the outside air by means of rubber tubing provided with a spring clamp *k*. It is obvious that water will enter the bath until the level rises above *d*, when the resultant negative pressure above the water in the bottle will stop its outflow at *b*. The bath is not perfectly automatic, because the difference in pressure in the bottle and the bath holds some water in *d*, which is released by momentarily

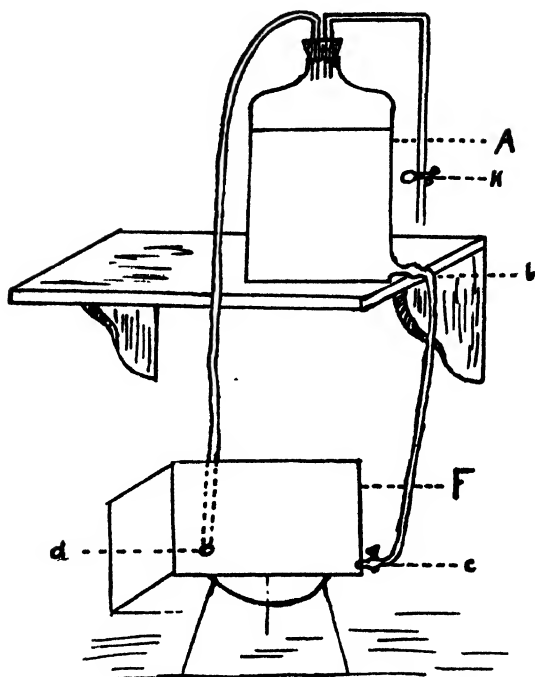


FIG. 1

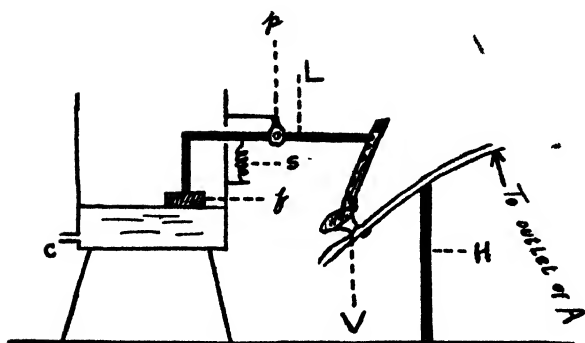


FIG. 2.

opening spring clamp k . The pressure is thus equalized, and the level of the water in the bath restored.

If the conditions demanded, it would be a simple matter to devise a strictly automatic mechanism.

As shown in figure 2 one might locate in one corner of the bath a wooden float f , connected with a lever L , working on a pivot p , on the outside surface of the bath's wall. With one end of the lever working against a light coil spring S , and the other regulating a valve V , controlling the air pressure over the water in A (fig. 1), the rise of the water in the bath would indirectly cut off the water supply, which would start to flow again when the evaporation of the water reversed the action of this mechanism. By making the length of the air valve lever adjustable, the level of the water in the bath could be changed at will. In such event stop-cock d of the bath as well as the opening in the bottle controlled by clamp k could be dispensed with. H represents a support for air tube leading to bottle A , figure 1.

A MODIFICATION OF THE WRIGHT-BUCHNER ANAEROBIC TUBE

RALPH R. MELLON

Department of Laboratories, Hahnemann Hospital, Rochester, New York

Received for publication. February 10, 1919

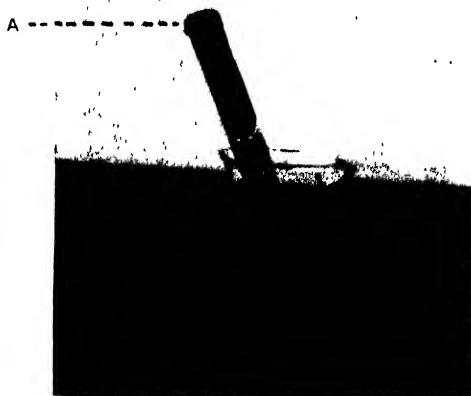
One of the early, anaerobic methods was that of Buchner which involved enclosing the culture tube in an outer airtight test-tube containing pyrogallic acid, employed of course as a reducing agent. Later Wright carried on the entire process in a single tube by depressing the cotton plug and filling the space above it with the acid, after which the tube was corked with a rubber stopper. The tendency for the pyrogallic acid to contaminate the slant and diffuse throughout a non-protein medium has been thought to detract from the value of the method.

With the object of still further simplifying the procedure and making it easier to handle, I have made the following alterations for my own work. I have found that the brand of cotton often employed in bacteriological work is unsuitable for the procedure owing to its permeability. What is known as the "Pearl" brand¹ has yielded most satisfactory results. The plug should be rolled so tightly that it can be tossed about if desired without losing its shape. The loose way in which many plugs are made adds to their permeability, and by no means favors their use in this method.

When the properly fashioned plug is inserted to within a short distance of the top of the slant, a file or some other instrument is run into its center to the depth of a few millimeters. I employ a 5-inch, 3-cornered file with a moderately sharp point about 1 cm. of which is bent at an obtuse angle. With the file in position an ordinary tallow candle is lighted, and paraffin is dropped on top of the plug to a depth of 2 or 3 mm. It should form a

¹Sold in Ann Arbor, Michigan, by Mack and Company.

layer on top of the plug. When the paraffin has set, pyrogallic crystals are lightly tamped into most of the remaining space. Warm 5 per cent NaOH is pipetted or poured over the crystals, and a soft rubber cap² is quickly slipped around the top of the tube, which is preferably a flanged one. A few drops of paraffin from the candle is then let fall on the junction of the tube and cap. With the slanting surface down, the tube is then inclined at a slight angle in the incubator. The inclination is adopted as an additional precaution, but is by no means necessary, for broth cultures can be grown in an upright position with ease



when using the same technique. Following removing of the cap and the pyrogallic residuum, one may open the tube by placing the file in the opening originally made by it in the center of the plug. The tube is heated gently, and the plug removed by spiral revolutions with the file. It is of advantage to discard this plug and use a fresh one to absorb the small amount of pyrogallic that may be present on the walls of the tube. This is usually essential with broth cultures.

²These caps are obtained from Parke, Davis and Company, Detroit, and are the same as used by them to cap their stock media.

The whole procedure need occupy but a few minutes. I have used the modification satisfactorily for the past five years. It is neat, clean and reliable. The paraffin coating over the cotton plug prevents the acid from immediately seeking the sides of the tube. It enters the plug slowly at one point only—the center—and when it ultimately permeates the remainder, the supply should be practically exhausted. Thus the tendency to drain down the walls of the tube is almost nil.

This tendency is further diminished by using an elastic rubber cap for a seal rather than a rubber stopper; as the latter, by its encroachment on the space of the tube increases somewhat the intratubal pressure. Furthermore this cap acts as a gauge for the success of the technique in the individual tube—anaerobiosis being indicated by the concavity developed on the caps' surface in response to the diminished intratubal pressure (see fig. at *A*). When blood-agar is used as a medium, its change in color is a sufficient index of successful reduction.

MODIFICATIONS IN THE TECHNIC OF DICK'S PLATE METHOD FOR ISOLATING ANAEROBES

ZAE NORTHRUP

Michigan Agricultural College, East Lansing, Michigan

Received for Publication, February 18, 1919

George F. Dick describes in a recent number of the *Journal of Infectious Diseases*, (23, 577) a very clever and simple method of growing and isolating anaerobes by a plate method. Briefly his method is as follows: Using ordinary petri dishes, the plates for isolating anaerobes are prepared and poured as with ordinary aerobic cultivations; they are then allowed to harden, and a second tube of the same medium is cooled to about 45°, and poured upon the hardened medium. While this latter layer is hardening, paraffin is heated to the smoking point, allowed to cool to about 45° and spread while warm with a spatula over the surface of the agar plate. For examining and fishing colonies developing on these plates Dick suggests that after the colony has developed, a little square of paraffin may be cut out above the colony, and lifted out.

The method of spreading the paraffin over the plates with a knife or spatula is unsatisfactory because it is too slow and is difficult to perform as the paraffin hardens quickly. When it is remembered that almost all strict anaerobes are spore formers there is no reason why the temperature of the paraffin should not be as high as 60° so that it may spread over the solid agar of its own liquidity; but the paraffin at 60° is cooled almost instantly by the cold agar, so there is but slight danger if any even to non-spore formers.

One of the first difficulties encountered after the plates had developed a day or so was the difficulty of seeing the colonies against the white background of the paraffin. Of course transmitted light proved to have advantages in locating the colonies

but it seemed that if a comparatively dark background could be furnished the colonies would be much more clearly visible. After trying numerous dyes, I found but one, Sudan III, a fat soluble red dye, which would dissolve in the liquefied paraffin without trouble, and produce a deep enough color to show up the colonies well. Scharlach R has not been tried but will probably work similarly. Chr. Hansen's butter color dissolves readily in the hot paraffin but the color, a deep orange, is not favorable for detecting colonies, and crystals of some sort are formed in the paraffin when it cools, which also is disadvantageous. Neither of the dyes used diffuses into the medium so that they have no antiseptic or other apparent effect on the cultures. A fat soluble dye of a deep blue, green or black color would be ideal for this use. A large amount of finely powdered charcoal added to the paraffin makes a fairly acceptable background.

Instead of cutting little squares out of the paraffin over the colony it is desired to fish, it has been found much more simple and less destructive to the colony to warm the entire edge of the petri dish slightly in the flame, run a sterile scalpel around the edge and remove the paraffin cover entirely. In this way the whole plate can be examined at leisure and if it is desired to keep this plate culture for future examination, the addition of paraffin may be repeated. When this modification of the original technic is used the addition of dye to the paraffin becomes less necessary.

The paraffin can be used over and over again, and if it becomes dirty from admixtures of colony growths it can be filtered while smoking hot. The paraffin used on the surface of the petri dishes should be perfectly clear as transmitted light as well as reflected light is often necessary for the examination of colonies.

The above modifications in technic have been used successfully in isolating *B. botulinus* from mixed cultures.

THERMOPHILIC BACTERIA

D. H. BERGEY

Laboratory of Hygiene, University of Pennsylvania

Received for publication, March 13, 1919

True thermophilic bacteria are those that grow at temperatures above the maximum temperature for the great majority of bacteria, especially the pathogenic forms. The maximum temperature for the pathogenic bacteria is about 45°C. Their optimum temperature is about 37.5°C. The true thermophiles show no growth, or only very feeble growth, below 40 to 45°C. Their development requires temperatures above 50°C., and some are able to develop at a temperature of 80°C., though most abundant growth is shown at 60 to 70°C.

A group of facultative thermophilic bacteria has been discovered which develop at room temperature, about 20°C., and have their optimum temperature at about 50°C., and their maximum temperature at about 60°C. In this group belong some of the well-known spore-forming soil organisms.

Two distinct types of true thermophilic bacteria have been found in the laboratory, viz., spore-forming and non-spore-forming rods, and thread forms belonging in the class of *Nocardia*.

Most of the thermophilic bacteria studied belonged to the spore-forming class and these varied in size of rods, size and location of the spores, and to a less degree in their biological characters.

The sources from which these bacteria are obtained indicate that this group is widely distributed in nature. They were found in the dust collected on apparatus in the laboratory, on clothes, in soil, in sewage, in the gastro-intestinal contents and feces of rabbits and guinea-pigs, on grain and in horse manure.

The isolation of these bacteria from the different materials is an easy matter, using the plate method with nutrient agar medium, the plates being incubated at a temperature above 50°C. Under these conditions the colonies developing on the agar medium are certain to be thermophilic and can be obtained in pure culture by transplanting on to tubes of slanted agar.

The biological characters of the 60 cultures studied showed marked variation, especially with regard to their action on gelatin, milk, starch solution and nitrate solution. Eleven cultures failed to show any liquefaction of gelatin others showed only slight liquefaction, while others showed complete liquefaction in three or four days. The action on the gelatin was ascertained by cultivating at 51° for three or four days, and then placing the tubes at a low temperature to note whether liquefaction had occurred.

Most of the true thermophilic cultures showed only slight acidity in litmus milk, though several showed distinct acidity with coagulation but no digestion of the casein. Most of the facultative thermophilic cultures showed a primary acidity with coagulation followed by a slightly alkaline reaction and digestion of the casein.

The action on starch varied in degree, as nearly all cultures appeared to have some action in splitting starch. A number of the cultures changed the starch to dextrin, maltose and glucose, though in many cultures no other change than the dextrin stage could be detected.

The reduction of nitrate showed marked variation in the different cultures. Some showed only slight evidence of nitrite formation, others showed marked nitrite formation, while some of the cultures reduced the nitrate to ammonia.

The true thermophiles belonging in the group of spore-forming bacteria differed in their morphologic and biologic character to such a degree that satisfactory classification was not possible.

An attempt has been made to classify the different types of thermophilic bacteria and a comparison of the results obtained showed that amongst the more than sixty cultures studied, at least seven different rod forms and one thread form were

encountered that may be classed as true thermophiles, while one type of rod was encountered that may be regarded as a facultative thermophile.

The characters of the nine different types studied are given in tabular form on page 304.

The facultative thermophiles studied appeared to be more closely related to each other in that they formed short chains of rods with rounded ends having small oval central spores. These cultures all liquefied gelatin, fermented starch, and reduced nitrate to ammonia. Their action on milk showed at first a slight acidity followed by a slight alkalinity and slow peptonization of the casein. These cultures appeared to belong to a single species.

The true thermophiles resisted a temperature of 100°C., to an unusual degree varying from two to eight hours, so that it appears probable that the optimum temperature for growth is related to the heat resisting powers of the spores.

The facultative thermophiles with their lower optimum temperature for growth also showed a lower resistance to the boiling temperature. This group of bacteria was killed in from one-fourth to one hour at 100°C.

Two cultures of thread-form organisms were studied which had thermophilic properties. These two cultures represented two distinct varieties in their biological activities. On agar they showed a distinct white, mealy growth after several days and this fact has led to their classification with *Nocardia*. These two cultures differed in their action on milk and on starch solution, the one did not change milk but fermented starch to maltose, the other produced an acid reaction in milk with coagulation and subsequent digestion of the casein but only very slight action on starch. Both cultures liquefied gelatin but neither reduced nitrate. The maximum temperature of growth was about 70°C., and both were killed at 100°C., in two hours.

Although the thermophilic bacteria are widely distributed in nature it is evident that their activities can be brought into play under exceptional conditions. These organisms have been found in thermal springs where temperature conditions

Thermophilic bacteria

	TRUE THERMOPHILES										FACULTATIVE
	Type 1	Type 2	Type 3	Type 4	Type 5		Type 6	Type 7	Type 8		Type 9
					Var. a	Var. b			Var. a	Var. b	
Source of organism.....	Dust and contaminated milk medium	Dust and soil	Dust, soil, horse manure	Dust, soil, horse manure	Dust, pig feces, horse manure	Dust, g cheese, g-pig feces	Horse manure	Horse manure	Rabbit's stomach	Contamination on agar	Dust, soil, horse manure
Form.....	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Thread	Thread	Rod
Motility.....	+	+	+	+	+	+	+	+	-	-	+
Size in microns.	0.6-0.8 x 3-4	0.5-0.7 x 2-3	0.6-0.8 x 4-5	0.6-0.8 x 2-4	0.2-0.4 x 2-3	0.2-0.4 x 2-3	0.5-0.6 x 2-2.5	0.3-0.4 x 1.5-2			0.5-0.7 x 2-3
Grouping.....	Chains	Chains	Singly	Singly	Singly	Singly	Singly	Singly			Short chains
Location of spores.....	Central	Central	Central	Polar	Polar	Polar	-	-	Conidia	Conidia	Central
Spores larger or smaller diameter than rods...	Smaller	Greater	Greater	Greater	Greater	Greater	-	-	+	+	Smaller
Gelatin liquefied	+	-	+	+	+	-	+	-	+	±	+
Nitrates reduced	NH ₃	N ₂ O ₅	±	N ₂ O ₅	N ₂ O ₅	N ₂ O ₅	±	N ₂ O ₅	±	±	NH ₃
Starch reduced.	+	±	+	+	±	±	-	-	+	-	+
Action in litmus milk.....	Aid coagulation	Slight acidity	Slight acidity	Slight acidity	Slight acidity	Slight acidity	Slight acidity	Slight acidity	Slight acidity	Acid coagulation and digestion	Acid coagulation and digestion

Minimum temperature.....	50-60	37	37-50	50	37	37	37	37	60	60	20-37
Maximum temperature.....	75-80	70	70-75	75-80	60-70	60	60	60	70	70	50-60
Thermal-death point at 100° C. in minutes.	400	300	200	180	120	60	5	5	120	120	15-60

favor their development. In the surface layers of the soil in the temperate and torrid zones the temperature frequently rises to a point where they can develop and it is probable that they find an important field for action under these conditions.

There are other conditions in nature where it is probable that these organisms find suitable environment for their activities. In the fermentation of silage temperatures have been recorded as high as 55°C., but more commonly the temperature ranges between 35 and 45°C. The production of heat is probably not due to chemical changes in the fodder, by the tissue cells, but primarily to the activities of the thermophilic bacteria. Under these conditions it seems likely that the facultative thermophiles begin the fermentation processes and when the temperature rises to a sufficient degree then the true thermophiles begin to develop. Their ability to act on carbohydrates and protein indicates that they probably find sufficient food for development in various fodder crops.

The spoiling of hay and other fodder crops and of grain, when insufficiently cured, is no doubt due largely to the action of the thermophilic bacteria. The charring effect noticed in piles of improperly cured hay and fodder and in manure is probably caused by the thermophilic bacteria.

The conditions of temperature under which the thermophilic bacteria thrive are paradoxical when we note that the optimum temperature of growth is at about the temperature at which ordinary egg and serum albumin begin to coagulate. The maximum temperature of growth is decidedly above the temperature at which egg and serum albumin coagulate. This phenomenal characteristic may be due to the reaction of the medium in which the bacteria are growing or to the mineral content of their own protoplasm.

The investigation was interrupted in 1917 on entering the government service and the cultures were discarded. This accounts for the lack of details that would be desirable but it was thought advisable to publish the results that had been noted.

STUDIES IN THE METABOLISM OF ACTINOMYCETES

II

SELMAN A. WAKSMAN

Department of Soil Bacteriology, New Jersey Agricultural Experiment Station

Received for publication December 2, 1918

4. THE GROWTH AND ACTION OF ACTINOMYCETES UPON EGG-MEDIA

Egg-media were made the subject of special study on account of the very excellent growth of the organisms upon them and also on account of the fact that the liquefaction of the medium is an indication of the proteolytic action of the organisms. Three media were used, namely:

1. Whole egg. The eggs were sterilized on the surface, contents transferred into sterile containers, mixed with sterile spatula, then tubed, slanted, coagulated and sterilized in the Arnold at 75° to 90° for three consecutive days.

2. Lubenau's egg-medium, consisting of a mixture of 4 to 6 volumes of thoroughly mixed eggs with 1 volume of nutrient broth containing 5 per cent of glycerin. Coagulation and sterilization as before.

3. Petroff's (1915) medium containing 2 parts of well beaten eggs, one part of meat juice (15 per cent glycerin) and gentian violet in strength of 1: 10,000. The medium was tubed, coagulated and sterilized as before.

A number of Actinomycetes isolated from the soil, wounds and other sources, and grown upon a synthetic medium, were inoculated upon these 3 media, which were then incubated at 37°. An excellent growth was obtained with nearly all the organisms in twenty-four to forty-eight hours.

The organisms grew almost equally well on all the three media, producing a similar growth and similar reactions, so that the first medium appeared just as satisfactory as the other two.

But, for the isolation of the organisms from wounds, the Petroff's medium would probably prove best since the gentian-violet would eliminate many contaminations. Glycerin which, as will be shown later, forms a good source of carbohydrate for these organisms, seems to be unnecessary in the egg-medium since the egg alone probably supplies all the necessary constituents for the organisms. Gentian-violet, in 1:10,000 concentration, has no inhibitive effect upon at least most Actinomycetes.

Without taking up the cultural description of the organisms upon the media, we might point out some characteristic chemical changes produced by the organisms. *A. madurae*, *A. griseus*, 128, *A. hominis* produced a liquefaction of the coagulated medium, indicating that these organisms are able to hydrolize the coagulated egg-albumin. It will be noticed that the organisms that are able to do this are the ones found to be most active proteolytically upon milk proteins and coagulated blood serum and which hemolized the blood in blood agar.

A second fact noted was the production of a deep brown to violet and almost black pigment by a number of species, such as *A. poolensis*, *A. bobili*, *A. viridochromogenus*, *A. reticuli*, 120, 205, *A. aureus*, *A. scabies*, 215 and 218. It will be observed that the organisms producing this brown to black pigment upon egg-media are the same ones that produced a brown pigment when grown upon milk. Pergolo (1917) stated recently that the ability of chromogenic bacteria to produce pigments when grown on Lubenau's medium is due to the presence of the yolk of egg; the ether-soluble part of the yolk favors the most chromogenesis. This is no doubt true. It is probable that these organisms produced the enzyme tyrosinase which acting upon the egg constituents (previously split) will result in the production of the pigment.

Summary

1. Egg-media allow a good growth of Actinomycetes to take place in a short period of time.
2. The addition of gentian-violet in a concentration of 1:10,000 to the medium has no detrimental effect upon these organisms.

3. The liquefaction of the coagulated egg is characteristic of the strongly proteolytic organisms.

4. The production of a brown pigment is characteristic of certain organisms.

5. THE ACTION OF ACTINOMYCETES UPON GELATIN

A solution of gelatin in water, with or without other nutrient constituents, is extensively used by bacteriologists to demonstrate the fact whether a certain organism is proteolytic in nature or not. The liquefaction of the gelatin is due to a proteolytic enzyme produced by the organism. Some investigators (Pollak) claimed that this enzyme, *gelatinase*, is distinctly different from other proteolytic enzymes since its action results only in the liquefaction of the gelatin, while its action upon the hydrolysis of proteins is very slight. Ascoli and Neppi (1908) have shown that the favorable action of the enzyme isolated by the method of Pollak (1905) upon the gelatin and not upon other proteins is not specific for the enzyme, but is due to the special conditions of the medium. The liquefaction of the gelatin has been usually taken as a sufficient index of the proteolytic action of the microorganisms or enzymes. Some investigators have gone further however and determined also the actual digestion of the gelatin by measuring the nitrogen precipitated by tannin, while others have obtained the amount of amino acid nitrogen produced, using the "formaldehyde" method of Sørensen.

Some of the Actinomycetes are found to be very active proteolytically. Attention has already been called to this fact in previous papers. Nearly all the Actinomycetes, with very few exceptions (*A. asteroides*), are found to liquefy gelatin at an earlier or later period of their growth, so that the liquefaction of gelatin as such could not be used for the differentiation of the species. Since they differ only in the rapidity of liquefaction, confusion would often arise, due to the fact that some forms were reported to be unable to liquefy the gelatin, while, had a longer period of incubation been allowed, the liquefaction would have been apparent.

Beijerinck (1900) demonstrated the fact that the *A. chromogenus* species produce *quinone* which acts upon the gelatin and converts it into an insoluble form. The production of a brown pigment upon the gelatin is due to the formation of quinone by the organisms in question.

Krainsky (1914) has recently shown that most Actinomycetes grow on gelatin media producing liquefaction in most cases; the chromogenus-species producing an insoluble gelatin compound. Waksman and Curtis (1916) have shown that all the Actinomycetes that they isolated from the soil liquefied gelatin but that the rapidity of liquefaction was distinctly different for different organisms. The production of a brown pigment was characteristic of some species.

The nature of the growth of the Actinomycetes upon gelatin, the chemical changes produced in the gelatin as a result of the growth of the organisms and the constancy of these characters for a given species will be reported on in this paper.

The cultures were the same studied in the previous investigations. The organisms were grown upon Czapek's solution agar, then inoculated upon the gelatin. One hundred and fifty grams of "Gold Label" gelatin were dissolved in a liter of distilled water, then tubed and sterilized in flowing steam on three consecutive days. The adjustment of the reaction did not seem to have an important effect upon the growth and liquefaction; but for the purpose of having conditions more constant, the pH of the gelatin solution was adjusted to 7.0 before sterilization, using the colorimetric method of Clark and Lubs (1917). At first the organisms were grown in colonies on plates and the diameter of the liquefied zone measured, but it was found that the growth in a standard size tube and subsequent measurement of the depth of liquefaction gave more comparable results. The latter method could not bring out the typical growth of the organism, but gave a good means for comparing the amount of liquefaction.

The results reported in table 1 include the information obtained three years ago with the same organisms, soon after their isolation from the soil, also the results of the action of the organisms upon gelatin after they had been grown for three years on artificial, mostly synthetic, culture media.

The incubation temperatures were 20° and 25°.

The amino nitrogen was determined at the end of forty days incubation, using the micro-method of Van Slyke (1914). In a case where the gelatin was not entirely liquefied, the liquid portion was used for the analysis.

A study of table 1 will reveal the fact that the liquefaction of the gelatin is a rather constant property of the organisms and can be used in their differentiation. But different methods and different temperatures of incubation may interfere with thoroughly concordant results. This is due to the fact that the different species will grow with a different degree of rapidity on different media and at different temperatures. The rapidity of liquefaction of gelatin may therefore be used only in supplying some additional information as to the identification of the organism, but may not be considered of absolute value. The production of a brown pigment, chiefly in the unliquefied portion of the gelatin, brought out more clearly by the use of the plate method, is more characteristic of the organism, but even here certain few organisms (*A. albus*) are found which originally produced a brown pigment but later lost that property, after they were grown for three years on artificial culture media. The production of aerial mycelial is also not absolutely constant. A few organisms may produce aerial mycelium at one time and not at another. These discrepancies can be readily explained by the variability of the same organism upon the same culture medium. If a large enough number of cultures of the same organism were studied, to eliminate the individual variation of the same strain on the same medium, these characteristics might become more definite although even then we would expect certain changes in the organism on transfer and continuous growth upon artificial culture media. But when we are studying only one or two tubes at a time, the possible error is very great. The organism cannot therefore be studied and identified on one or two media, most of which, as commonly used by bacteriologists and plant pathologists, are not standard in composition, such as nutrient agar, cornmeal agar, etc. A large amount of information has to be obtained for each individual organism, grown on standard media and studied in detail as to its biochemical characteristics,

TABLE 1
The growth of actinomycetes upon gelatin

NAME OF ORGANISMS	FALL 1915, 20°			FALL 1918, 20°				FALL 1918, 25°		
	Liquefac- tion	Pigment	Aerial mycelium	Liquefac- tion mm. in 30 days	Pigment	Aerial mycelium	NH ₄ N, mgm. in 10 cc. Control= 7 25	Liquefac- tion mm. in 4 days	Pigment	Aerial mycelium
<i>A. violaceus-ruber</i>	Slow	None	White	20	None	None	36.0	8	Bluish	None
<i>A. violaceus-caesari</i>	Rapid	None	None	8	None	None	21.0	10	None	None
<i>A. californicus</i>	Slow	None	White- gray	6	None	Gray	18.2			
<i>A. scabies</i>	Rapid	Brown	None	10	Brown	None	42.0	12	Brown	None
<i>A. exfoliatus</i>	Slow	None	Gray- brown	10	None	Brown	46.2	12	None	Gray
<i>A. diastaticus</i>	Rapid	None	White	40	None	White	50.0	20	None	White
<i>A. albus</i>	Rapid	Brown	White	5	None	White		4	None	White
<i>A. reticuli</i>	Rapid	Brown	None	12	Brown	White		2	Brown	White
<i>A. 120</i>	Rapid	Brown	Pink	36	Brown	Pinkish		30	Brown	Pink
<i>A. citreus</i>	Rapid	None	White	9	None	None	31.5	2	None	None
<i>A. griseus</i>	Rapid	None	White	50	None	White	54.6	25	None	White
<i>A. alboflavus</i>	Rapid	None	None	5	None	None		15	None	None
<i>A. verne</i>	Rapid	None	None	12	None	None	14.4	10	None	None
<i>A. albosporus</i>	Rapid	None	Reddish	34	None	Reddish		12	None	Reddish
<i>A. bobili</i>	Rapid	Brown	None	35	Brown	None	51.0	8	Brown	None
		(late)								
<i>A. lipmanji</i>	Rapid	None	White- gray	10	None	None	32.5	10	None	None
<i>A. rudgersensis</i>	Rapid	None	None	30	None	None	33.5	5	None	White
<i>A. aureus</i>	Rapid	Brown	White	4	Brown	None	14.8	5	Brown	None

<i>A. fradii</i>	Rapid	None	White	4	None	White (scarce)	16 8	8	None	None
<i>A. lavendulae</i>	Slow	Brown	None	None	Brown	None		None	Brown	None
<i>A. maduræ</i>				36	None	None	43.2	10	None	White
<i>A. hominis</i>				16	None	None	51 0	10	None	None
<i>A. bovis</i>				30	None	None	19 2	10	None	None
<i>A. asteroides</i>				None	None	None		None	None	None
96.....				13	Golden	White		20	Golden	White
128.....	Rapid	None	White	18	None	Ash-gray	45.5	20	None	Gray
161.....	Slow	None	White	Start	None	White		None	None	White
202.....				6	Brown	None		3	Brown	None
101.....	Medium	Brown	Green	10	Brown	Green	40 0	2	Brown	Green
205.....				15	Brown	White	30 0	10	Brown	White
206.....				40	None	None		16	None	None
215.....				6	Brown	Scant		20	Brown	None
214.....				20	None	None		25	None	None
<i>A. poolensis</i>				8	Golden	None	33.0	10	None	None
168.....	Medium	Brown	White	15	Red	White	35.5	8	Brown	White

and even then we will have to study species not by themselves but in their group relationship.

The amino nitrogen content of the liquefied gelatin points distinctly to the proteolytic properties of the organisms. Although at 25° nearly all the cultures, with the exception of *A. lavendulae*, *A. asteroides*, *A. aureus*, 96, and one of two others, not reported in the Table, were fully liquefied in twelve to twenty-five days, the amino nitrogen content of the liquefied gelatin was distinctly different. This indicates that, although some organisms may liquefy the gelatin, the hydrolysis is not carried very far. For example, the gelatin of *A. violaceus-caesari*, *A. verne*, *A. bovis*, *A. aureus*, and *A. fradii* cultures shows a comparatively small increase in the amino nitrogen content of the gelatin, while the cultures of *A. griseus*, *A. diastaticus*, *A. exfoliatus*, *A. bobili*, *A. madurae*, *A. hominis*, 128 contain large quantities of amino nitrogen. These results, when compared with those reported in the previous papers, indicate that the organisms most active proteolytically on milk and blood serum are also most active upon gelatin, while those least active upon those natural protein-rich media are among the least active on gelatin.

These results would seem to point to the fact that the liquefaction of gelatin may not necessarily indicate a strong proteolytic action, although the proteolytically active organisms liquefy gelatin very rapidly, due to the hydrolysis of this compound. Before we know more definitely the physical process of gelatin liquefaction and the accompanying chemical changes, if there are any (as in the case of warming the gelatin), we cannot expect to correlate the hydrolysis and liquefaction of gelatin in more than a probable way. Berman and Rettger (1918) have recently stated that the ability of an organism to liquefy gelatin is not in itself a proteolytic or even a gelatinolytic function, for some organisms which can liquefy gelatin are unable to carry the change beyond the gelatose stage, and fail to decompose "purified" proteose and casein.

The production of enzymes liquefying and hydrolyzing gelatin, goes on even when the organisms are grown upon synthetic media. Several Actinomycetes were grown on Czapek's

synthetic solution, then filtered. Both filtrate and mycelium, treated by the "acetonedauerhefe" method, were able to liquefy the gelatin in twenty-four to ninety-six hours with an increase in the amino nitrogen content.

The organisms producing the brown pigment upon gelatin usually produce a similar pigment when grown upon other complex nitrogenous media, such as egg, blood and blood serum medium. This is probably due to the production of an oxidizing substance such as quinone demonstrated by previously named investigators upon gelatin. The action of these organisms upon gelatin results first in a slow liquefaction of the gelatin, with a small increase in the amino nitrogen content, indicating hydrolysis. Later, particularly with the production of an intense brown pigment and not at a very high temperature (about 20° or below), the liquefied gelatin solidifies again, as in the case of a few chromogenus species, including *A. lavendulae* and 205.

6. THE UTILIZATION AND TRANSFORMATION OF DIFFERENT CARBON COMPOUNDS BY ACTINOMYCETES

Several important references are found in the literature on the utilization of carbon compounds by Actinomycetes. Beijerinck (1900) stated that these organisms produced diastase; the production of this enzyme was also demonstrated by Sames (1900), starch being reduced only to the point of giving the erythro-reaction and no reducing sugars being produced. Caminiti (1913) found that the Actinomycetes produced invertase and diastase. Münter (1913) made a rather thorough study of the utilization of carbon compounds using ammonium nitrate as a source of nitrogen. Glycerin, levulose, glucose, galactose, mannite, starch and lactose proved to be good sources of carbon for all Actinomycetes; only one organism made a poor growth on lactose; arabinose was well utilized by some and not by others; sucrose was used only by certain species (chromogenus types and one other); only one organism could assimilate inulin. Oxalic, tartaric and hippuric acids in the form of sodium or calcium salts, were utilized to only a very small extent or not at

all, while succinic and citric acids offered good sources of carbon. With the introduction of the oxy groups into the molecule of succinic acids, its utilization was found to decrease since the growth diminished with malic acid and almost ceased entirely with tartaric acid. Acetic acid was utilized to some extent, while lactic acid formed a good source of carbon.

Krainsky (1914) stated that Actinomycetes made a good growth upon media containing mono-, di- and poly-saccharides and salts of organic acids. CaCO_3 was produced from glucose and calcium malate. Glucose was oxidized with the production of CO_2 , leaving the medium acid. A few species were found by Krainsky to produce invertase and most of them produced a rather active diastase, while maltose and raffinose were not split. Cellulose was used by the Actinomycetes with different rapidity.

Salzmann (1902) obtained a good growth of *Actinomyces odorifer* upon many carbohydrates, particularly glycerin, in neutral, acid or alkaline solutions. The calcium salts of organic acids containing one carboxyl group proved to be poor sources of carbon, while good growth took place, when a second carboxyl group was present.

Fousek (1912) stated that glucose, lactose, starch and cellulose formed good sources of carbon for Actinomycetes, while urea and uric acid could not be used as sources of carbon, although they were good sources of nitrogen.

The fermentation of carbohydrates is commonly used by bacteriologists as a method of differentiating different bacterial species and even groups. The information thus obtained together with a mere microscopic and macroscopic study is often sufficient for a proper identification. The change in reaction (usually acid production) and production of a gas are taken as indications of the fermentation, the two reactions not necessarily going hand in hand.

To find out whether this method could also be applied to the study of Actinomycetes, the ordinary medium and bacteriological methods were used at first. Sterile beef infusion (500 grams beef per liter of water, boiled, filtered and sterilized) was inoculated with *B. coli-communior* and incubated at 37° for twenty

hours. The material was then boiled for ten minutes, adjusted to pH = 7.6 to 7.7, boiled again, filtered and sterilized. One per cent of the proper carbohydrate and 1 per cent of Andradé (Holman, 1914), indicator were added to the sterile sugar-free broth, and the medium was then distributed in fermentation tubes. The tubes were steamed in the Arnold for thirty minutes, incubated to insure sterility and inoculated with the Actinomycetes. This method of procedure, so much used in bacteriological work, proved to be unsatisfactory for the study of the Actinomycetes. These organisms do not form any gas from the different carbohydrates, therefore the fermentation tube is unnecessary; they do not produce any acid, as will be shown elsewhere, but have a tendency to produce a decrease of the hydrogen-ion concentration of the medium, therefore the Andradé indicator, which registers only acid production is of no value in this case. Furthermore, since the meat infusion itself will allow a growth of the Actinomycetes, the effect of the carbon compounds cannot be differentiated properly.

An inorganic medium standard in composition had therefore to be selected for this study. Since the Czapek's solution agar had been extensively used for the study of the Actinomycetes by the writer and found to give satisfactory results, this solution was also used as a basis for the following experiments. One gram K_2HPO_4 , 0.5 gram $MgSO_4$, 0.5 gram KCl , 0.01 gram $FeSO_4$, and 2 grams $NaNO_3$ were added to every liter of distilled water. This was used as a basis to which 2 per cent of the following carbon compounds were added: arabinose, glucose, lactose, sucrose, maltose, mannite, glycerin, starch and the sodium salts of acetic, malic, tartaric, oxalic and lactic acids. All of the materials were chemically pure, either Kahlbaum's or Merck's. Cellulose was added in two forms: a small piece of Whatman filter paper was introduced into each tube, the solution containing no other source of carbon, or 1 cc. of 0.5 per cent cellulose suspension prepared by the method used by McBeth (1916) and others. The media were tubed, sterilized at 15 pounds pressure for fifteen minutes, incubated to insure sterility and inoculated with several cultures of Actinomycetes, grown on the

Czapek's agar. The cultures were incubated for fifteen days. At the end of that period the cultures were studied as to the growth characters and the effect of different carbon compounds upon the change of reaction, as measured by the hydrogen-ion concentration, using the phenol-sulphonphthalein series of indicators of Clark and Lubs (1917), and upon the production of nitrites; these were determined by the Griess colorimetric method (1879).

The results obtained from the study of 27 organisms using 14 different carbon compounds were tabulated. The media to which arabinose, glucose, maltose and lactose were added were distinctly acid due to the effect of the high pressure in sterilization upon these sugars.

The results presented in table 2 yield themselves readily to comparisons. It might be argued that since the hydrogen-ion concentration of the arabinose, glucose, maltose and lactose media was higher than that of the others, being distinctly acid, due to the effect of sterilization upon these sugars, the comparative values may be affected. But this factor is not of very great importance, as will be pointed out in one of the following papers of this series, if the reaction does not extend beyond certain limits of acidity and alkalinity. It was also recently demonstrated by Gillespie (1918) that, although on media with an hydrogen-ion concentration of $\text{pH} = 5.2$ and less the growth of *Actinomyces chromogenus* (*A. scabies*) was slower and generally less vigorous than in the less acid media, the difference between the media having an hydrogen-ion concentration of $\text{pH} = 5.8, 6.1$ and 7.2 was hardly noticeable. The hydrogen-ion concentration of the arabinose, glucose and lactose media was about $\text{pH} = 6.2-6.3$, so that the effect of the reaction would probably be of no great importance in this case.

Upon adding the figures designating the amount of growth for all the organisms studied and dividing the sum by the number of organisms, we obtain certain factors, which designate the utilization of the different carbon compounds by the Actinomycetes as a group. The factors are for arabinose 1.13, glucose 3.0, lactose 2.69, sucrose 1.93, maltose 2.67, mannite 2.17, glycerin 2.37,

TABLE 2

The utilization of different carbon compounds by actinomycetes and the effect of these upon the hydrogen ion concentration of the medium and reduction of nitrates

NAME OF ORGANISMS	ARABINOSE				GLUCOSE				LACTOSE				SUCROSE				MANNITE			
	Gr	AM	Colt	pH	Nit	Gr	AM	Col	pH	Nit	Gr	AM	Col	pH	Nit	Gr	AM	Col	pH	Nit
Control.....	0	0	0	6.2	5	0	0	0	6.2	0	0	0	0	0	0	0	0	0	7.2	0
<i>A. violaceus-ruber</i>	1	0	1	6.3	5	0	2	1	7.0	5	5	0	0	0	0	0	0	0	7.2	5
<i>A. violaceus-caesari</i>	1	0	1	6.3	1	3	0	1	6.4	0	1	0	2	6.3	0	2	0	2	6.9	1
<i>A. albus</i>	2	0	1	6.4	0	3	2	1	6.4	0	3	2	1	6.9	1	2	0	1	6.9	1
<i>A. aureus</i>	0	0	0	6.2	1	4	1	1	6.5	1	3	2	1	6.4	1	2	0	2	7.4	0
<i>A. esfoliatus</i>	4	2	1	6.8	0	4	2	1	7.0	0	4	2	1	6.8	1	5	3	1	7.1	1
<i>A. griseus</i>	3	2	1	6.5	0	4	2	2	6.9	2	3	2	2	7.1	0	1	2	2	7.4	0
<i>A. scabies</i>	4	2	1	6.7	1	2	0	1	6.4	0	4	1	1	6.9	1	2	0	1	6.9	1
<i>A. verne</i>	0	0	0	6.2	0	1	0	2	6.3	1	2	0	1	6.4	3	2	0	2	6.8	3
<i>A. lipmanti</i>	0	0	0	6.2	0	1	0	2	7.0	0	3	3	2	7.0	2	3	0	3	6.8	0
<i>A. virido-chromogenus</i>	3	2	2	6.7	2	4	3	2	7.1	2	3	3	2	6.9	3	2	3	2	7.3	2
<i>A. bobili</i>	0	0	0	6.2	0	2	0	2	6.5	0	3	0	1	6.7	1	2	0	2	6.9	1
<i>A. poolensis</i>	0	0	0	6.2	0	1	0	2	6.4	0	2	0	2	6.8	0	1	0	2	7.0	1
<i>A. diastaticus</i>	4	2	3	6.9	1	4	2	2	7.1	0	4	4	2	6.8	1	2	2	1	7.4	0
<i>A. fradii</i>	3	1	3	6.5	2	4	2	2	6.5	0	3	4	1	6.8	1	1	0	2	7.3	1
<i>A. roseus</i>	1	2	0	6.4	4	3	0	1	7.1	5	1	2	0	2	6.7	1	2	1	7.3	3
<i>A. albosporus</i>	0	0	0	6.2	0	3	2	1	6.5	1	2	2	1	6.5	1	2	0	1	7.0	0
<i>A. asteroides</i>	0	0	0	6.2	0	4	0	1	6.7	3	2	2	1	7.0	2	2	0	2	6.8	2
<i>A. madurac</i>	0	0	0	6.2	0	0	0	0	0	0	0	1	0	2	7.6	1	0	0	6.4	1
<i>A. hominis</i>	0	0	0	6.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>A. bovis</i>	0	0	0	6.2	0	0	0	0	0	0	0	2	2	7.3	0	2	0	2	7.6	1
104.....	0	0	0	6.2	0	1	0	2	6.4	0	1	0	2	6.3	0	1	0	2	7.6	0
120.....	0	0	0	6.2	0	4	3	2	6.9	4	2	0	1	6.3	1	1	0	2	7.0	1
128.....	0	0	0	6.2	0	2	2	1	6.4	0	2	0	1	6.4	0	2	0	2	7.0	1
154.....	0	0	0	6.2	0	3	0	1	6.4	0	4	0	1	6.4	0	2	1	1	7.0	1
168.....	0	0	0	6.2	0	4	2	1	7.2	2	4	2	1	7.0	1	2	1	1	7.0	1
205.....	0	0	0	6.2	0	1	0	2	6.3	0	2	0	2	6.3	0	2	0	2	6.8	0
215.....	1	0	2	6.4	4	3	2	1	6.4	1	3	2	1	6.9	3	3	1	1	7.2	1

* Gr = growth; AM = aerial mycelium; Col = colonies; pH = index of hydrogen ion concentration; Nit = nitrates; 0 = absence; 1 = faint or trace; 2 = fair; 3 = good; 4 = very good; 5 = excellent.

† 1 Type = round colonies; 2 type = floccules; 3 type = mass of growth; 2 for the organic acids indicates a small amount of growth on the bottom of tube.

TABLE 2—Continued

NAME OF ORGANISMS	GLYCERIN				STARCH				CELLULOSE				CELLULOSE SUSPENSION				MALTOSÉ			
	Gr	AM	Col	pH	Nit	Gr	AM	Col	pH	Nit	Gr	AM	Col	pH	Nit	Gr	AM	Col	pH	Nit
	0	0	0	7.1	0	0	0	0	7.1	0	0	0	0	7.1	0	0	0	0	6.2	0
Control.....	3	1	1	6.7	5	5	2	1	7.0	5	2	0	0	1	2	3	4	2	1	7.2
<i>A. violaceus-ruber</i>	0	0	0	7.4	0	3	0	1	7.0	1	2	0	0	1	2	3	0	2	5	6
<i>A. violaceus-caesari</i>	3	1	1	7.1	0	3	1	1	7.2	1	0	0	0	7.3	0	0	0	2	6	0
<i>A. albus</i>	3	1	1	7.0	1	2	1	1	7.1	1	0	0	0	7.0	0	0	0	2	6	0
<i>A. aureus</i>	3	1	1	7.0	0	4	2	1	7.3	1	0	0	0	7.0	0	0	0	3	6	3
<i>A. erfoliatus</i>	2	1	2	7.2	1	4	2	2	7.4	2	1	0	0	7.6	0	1	0	3	7	6
<i>A. griseus</i>	3	0	1	7.4	2	3	1	1	7.4	1	1	0	0	7.4	0	2	0	2	6	0
<i>A. scabies</i>	1	0	2	7.0	1	1	0	2	6.9	1	3	0	0	7.2	1	1	0	2	6	2
<i>A. verne</i>	3	2	2	7.5	3	3	2	2	7.3	4	0	0	0	7.4	1	3	0	1	5	6
<i>A. lipmanii</i>	2	2	2	7.3	3	3	2	2	7.5	2	1	1	1	7.1	1	3	0	2	6	2
<i>A. virido-chromogenus</i>	3	0	1	7.4	1	3	0	1	7.1	1	3	0	0	7.4	0	0	0	2	6	0
<i>A. bobli</i>	4	0	1	7.2	1	3	0	1	7.3	3	2	0	0	7.1	0	0	0	2	6	4
<i>A. poolensis</i>	4	4	2	7.4	1	4	1	2	7.5	1	0	0	0	7.1	0	0	0	3	8	0
<i>A. diastaticus</i>	3	1	1	7.2	1	4	1	1	7.1	2	0	0	0	7.5	0	0	0	2	6	2
<i>A. fradii</i>	2	1	0	7.1	0	4	3	0	1	7.0	5	1	0	7.7	4	0	0	2	6	0
<i>A. roseus</i>	1	0	2	7.1	0	4	0	1	7.5	3	0	0	0	7.2	0	0	0	2	6	6
<i>A. albosporeus</i>	2	1	1	6.7	4	0	0	0	6.9	0	1	0	1	6.7	1	1	0	2	6	2
<i>A. asteroides</i>	1	0	2	7.2	1	0	0	0	0	0	0	0	0	7.1	1	1	0	2	6	2
<i>A. maduræ</i>	5	3	1	7.6	3	0	0	0	0	0	0	0	0	7.5	0	4	3	1	7	4
<i>A. hominis</i>	1	2	7.2	5	0	0	0	0	0	0	0	0	0	7.2	0	1	0	2	6	5
<i>A. bonis</i>	1	0	2	7.0	0	1	0	2	6.8	0	1	0	1	7.1	0	1	0	2	6	0
104.....	3	1	3	7.3	1	5	2	2	7.0	5	3	0	0	1	1	1	0	2	6	2
120.....	1	1	1	7.1	0	3	1	1	6.8	0	1	0	1	7.0	0	0	4	2	1	7
128.....	2	0	2	7.2	1	2	0	1	7.1	1	2	0	0	7.2	0	0	3	4	1	6
154.....	4	1	1	7.5	4	3	1	1	7.2	1	2	0	0	7.1	0	1	0	2	6	2
168.....	1	0	2	7.0	1	4	1	1	7.3	1	2	0	0	7.3	0	4	3	2	1	6
205.....	3	1	1	7.3	1	4	2	2	7.6	1	2	0	0	7.1	1	1	1	1	7	0
215.....	3	1	1	7.3	1	4	2	2	7.6	1	2	0	0	7.1	1	1	1	1	7	0

TABLE 2—Continued

NAME OF ORGANISMS	SODIUM ACETATE				SODIUM LACTATE				SODIUM TARTRATE				SODIUM OXALATE				SODIUM MALATE			
	Gr.	AM	Col.	pH	Nit.	Gr.	AM	Col.	pH	Nit.	Gr.	AM	Col.	pH	Nit.	Gr.	AM	Col.	pH	Nit.
Control.....	0	0	0	7.3	0	0	0	0	7.3	0	0	0	0	7.8	0	0	0	7.8	0	0
<i>A. violaceus-ruber</i>	1	0	2	7.8	5	1	0	2	7.8	1	0	0	0	7.7	2	1	0	2	8.1	3
<i>A. violaceus-caesari</i>	1	0	2	7.8	0	1	0	2	6.6	1	1	0	2	7.6	0	1	0	2	8.0	1
<i>A. albus</i>	1	0	2	7.7	0	2	0	2	7.0	1	0	0	2	7.8	1	1	2	7.6	0	1
<i>A. aureus</i>	1	0	2	7.0	0	1	0	2	6.2	0	0	0	2	7.7	0	0	0	7.6	0	0
<i>A. exfoliatus</i>	1	0	2	7.1	0	1	0	2	6.4	0	2	0	2	7.6	0	1	0	2	7.8	0
<i>A. griseus</i>	1	0	2	8.2	0	1	0	2	6.6	0	1	0	2	7.4	1	1	0	2	7.9	0
<i>A. scabies</i>	1	0	2	7.0	0	1	0	2	6.6	0	1	0	2	7.5	0	1	0	2	7.6	0
<i>A. verne</i>	1	0	2	7.0	0	1	0	2	7.0	1	0	0	2	7.7	1	0	0	2	7.6	0
<i>A. lipmanii</i>	1	0	2	7.5	2	2	0	1	6.8	0	2	0	1	7.8	1	1	0	1	7.7	1
<i>A. virido-chromogenus</i>	2	0	2	7.4	0	1	0	2	6.6	1	0	0	0	7.6	0	1	0	2	8.0	0
<i>A. bobii</i>	0	0	7	3	0	0	0	2	6.6	0	0	0	0	7.5	0	1	0	2	8.1	0
<i>A. poolensis</i>	0	0	7	6	0	1	0	2	6.6	0	0	0	0	7.5	0	1	0	2	8.1	1
<i>A. diastaticus</i>	1	0	2	7.3	0	1	0	2	6.6	0	1	0	2	7.8	0	0	0	2	8.0	0
<i>A. fradii</i>	1	0	2	7.3	0	1	0	2	6.6	0	1	0	2	7.7	1	1	0	2	8.0	0
<i>A. roseus</i>	1	0	2	7.3	1	1	0	2	6.5	1	1	0	2	7.7	1	1	0	2	7.8	1
<i>A. albosporus</i>	1	0	2	7.6	2	2	0	2	7.7	4	2	2	1	7.5	2	1	0	2	7.7	2
<i>A. asteroides</i>	1	0	2	7.4	1	2	1	1	6.6	1	1	0	2	7.7	1	1	0	2	8.0	1
<i>A. maduræ</i>	1	0	2	7.5	0	0	0	2	7.8	1	1	0	2	7.8	1	1	0	2	8.6	1
<i>A. hominis</i>	1	0	2	7.4	2	0	0	2	7.6	0	0	0	2	7.7	1	1	0	2	8.0	0
<i>A. bovis</i>	1	0	2	7.7	0	1	0	2	7.7	0	0	0	2	7.5	0	0	0	2	8.2	0
104.....	2	0	1	8.0	1	1	0	2	6.6	1	1	0	1	7.7	1	1	0	2	8.3	1
120.....	1	0	2	7.3	1	1	0	2	6.6	0	1	0	2	7.7	0	1	0	2	8.0	0
128.....	0	0	7	4	0	1	0	2	6.6	0	1	0	2	7.8	0	1	0	2	7.6	0
154.....	1	0	2	7.4	0	2	0	2	6.6	0	2	0	2	7.7	0	1	0	2	7.7	0
168.....	1	0	2	7.9	1	0	0	2	6.6	0	0	0	2	7.6	0	1	0	2	7.6	0
205.....	2	0	1	8.0	2	1	0	2	6.6	0	1	0	2	7.8	1	1	0	2	7.8	1
215.....	2	0	1	8.0	2	1	0	2	6.6	0	1	0	2	7.8	1	1	0	2	7.8	1

starch 3.08, cellulose paper 1.25, cellulose suspension 0.75, sodium acetate 1, sodium lactate 1.16, sodium tartrate 0.88, sodium oxalate 0.79, sodium malate 1.00. Although these factors are not absolute in value, since they are affected not only by the presence or absence in the investigation of different individual organisms, but also by the periods of incubation and individuality of the recorder as well as by the inexact and abstract value of the estimate of growth. But the facts that these organisms were selected at random, that many representatives of the group were taken, a similar treatment used, and record of the amount of growth carefully made by one person at one time, will tend to give a certain value to these indications.

Starch was found to give the highest figure and it probably is one of the best sources of energy for most Actinomycetes. Glucose and lactose follow next, while maltose and glycerin occupy only the fourth and fifth places. That glycerin was not found to be such a good source of carbon for the Actinomycetes, as starch, glucose and lactose, may be due also to the source of nitrogen which affects the utilization of the carbohydrates, as was pointed out by Krainsky (1914), a point which will be taken up later.

Sucrose, cellulose, both paper and suspension, arabinose and the sodium salts of organic acids are rather poor sources of energy for the group as a whole as indicated by the factors of growth with NaNO_3 as a source of nitrogen. But, on comparing the action of individual organisms upon these carbon compounds, we find important differences between the sodium salts of the organic acids on the one hand and sucrose, arabinose and cellulose on the other. The sodium salts of the organic acids, particularly some of them, such as the malate, lactate and acetate can be used to some extent; the growth of the organisms upon these carbon compounds is very restricted indicating the fact that they do not offer very favorable carbon sources for the Actinomycetes. The growth of most organisms upon the liquid media containing these organic acids was limited to minute colonies or flakes in the bottom of the tubes, and only in few instances was a fair amount of growth obtained.

The amount of growth of an organism, with sucrose as a source of energy, depends entirely upon the ability of that organism to produce invertase, although even the organisms not producing invertase seem to use it to some extent. Cellulose and arabinose are used by only a few organisms, and this leads to the reduction of the growth factor for the whole group, although, when an organism does use these compounds, the growth may be quite abundant.

The reduction of nitrates to nitrites is a rather common property of the Actinomycetes, but the extent of reduction and even the very presence of it seems to depend on the nature of the carbon source present in the medium. When the figures representing the different quantities of nitrites produced (and these are again only relative) by the different organisms are added and the sum divided by the number of organisms, we obtain a factor for nitrite production by Actinomycetes for each source of carbon. These are for arabinose 0.92, glucose 1.13, lactose 1.42, sucrose 1.07, maltose 1.5, mannite 1.5, glycerin 1.66, starch 1.79, cellulose paper 0.54, cellulose suspension 0.46, acetate 0.83, lactate 0.53, tartrate 0.52, oxalate 0.5 and malate 0.84. Although the same criticisms can be applied to this method as to the measurement of the utilization of carbon compounds, the results obtained point to the fact that the method is not so inaccurate, when a large enough number of organisms are studied. A rather fair parallelism is obtained between the availability of the carbon compound as indicated by the growth of the organisms and the reduction of nitrates, pointing to the fact that the reduction is most active with the best growth, in the presence of a readily available carbon compound. Starch is again leading and is followed by glycerin, maltose, mannite, lactose and glucose. The interesting thing is the interchange of places between glycerin and glucose; while the growth factor of glucose was second and of glycerin fifth, the nitrite factor of glycerin was second and of glucose sixth. Arabinose, cellulose and the salts of the organic acids came last. The salts of the organic acids which gave higher growth factors gave also (except lactate) higher nitrite factors. This indicates that the process of nitrate

reduction is not a part of the energy utilization process, but accompanies the energy transformation of the organism. This question will be taken up in detail in a paper on nitrogen transformation by Actinomycetes.

As to the question of change in reaction, attention may be merely called here to the fact that the Actinomycetes, with very few exceptions, seem to be active in producing alkalinity in the medium, when NaNO_3 is the only source of nitrogen. An increase in acidity is observed only in few instances, while an increase in alkalinity is very apparent for most organisms. When we calculate the average for the hydrogen-ion concentration of the different organisms for one carbohydrate, we find that the organisms were most active in this respect in the starch medium. This seems to correspond with the occurrence of the best growth and largest quantities of nitrites. The organisms growing most rapidly, in the presence of a readily available carbon compound, reduce the nitrates very actively.

The source of carbon affects the pigment of the colony; thus, for example, the colonies of *A. fradii* are yellow in starch and arabinose, orange colored in glucose and white in glycerin. Having discussed the utilization of carbon compounds as a whole, we will take up now the specific action of Actinomycetes upon the three carbohydrates, sucrose, starch and cellulose.

Sucrose is used to some extent by nearly all the Actinomycetes studied and it forms the only source of energy in the Czapek's solution agar, used extensively by the writer for the study of this group of organisms. Only those organisms that produce invertase can make on this medium more than a rather poor growth. This fact is of great importance in the differentiation of the Actinomycetes, since media containing readily available constituents, such as nutrient agar, etc., allow these organisms to develop rapidly and very little differentiation can be made between the different organisms. A characteristic aerial mycelium is usually produced on the Czapek's solution agar, which is also very important in the identification of an organism. When glucose, starch or glycerin are substituted in place of sucrose in this medium, the growth of most organisms is much

quicker and more abundant, but in most cases not as characteristic. Only a few of the organisms were found to be able to invert the sucrose of the medium: these are *A. violaceus-caesari*, *A. verne*, *A. scabies*, *A. bobili*, 215 and 104. All these organisms, with the exception of the last make a very abundant growth upon the Czapek's solution agar, while the growth of the other organisms is restricted and aerial mycelium is produced early and readily. We can thus see that the amount of growth of an organism and the corresponding production of aerial mycelium may not be anything else but a result of the nutrition of the organism.

Two methods were used for the study of diastase production, the tube and plate method. In the first case 10 grams of starch were boiled for thirty minutes in distilled water. Upon the addition of the minerals of the Czapek's solution (all constituents except sucrose), the volume was made up to one liter and distributed into equal sized test tubes, 10 cc. to each tube. On sterilization, the tubes were inoculated with the different Actinomycetes and both inoculated and uninoculated tubes (for control) were incubated at 25°C. At the end of fourteen to fifteen days, the tubes were taken out of the incubator. The reading was made as follows: One control tube (uninoculated) was kept side by side with the tube containing the organism and the difference in the heights of starch in the two tubes was taken as the diastatic action of the organism. This method has the advantage over the plate method that different layers of the liquid in the tube can be pipetted off and tested for the presence of dextrans and reducing sugars.

The plate method consists in adding 1.2 per cent agar to the liquid medium and pouring 10 to 12 cc. of the sterile liquefied agar into sterile Petri dishes. Upon cooling, these are inoculated and incubated at 25° for fifteen days. At the end of that period, the surfaces of the plates are covered with a solution of iodine and potassium iodide. The clear zone around the growth indicates the diastatic action. The results of both methods are given in table 3. Plate I shows typical colonies with the diastatic zone which can be differentiated from the growth itself.

The two methods correspond fairly well, although they do not go hand in hand. But, with either method, the active diastase production by Actinomycetes is evident. With the exception of *A. asteroides*, all the other organisms hydrolize the starch very rapidly. *A. scabies* produced some hydrolysis as shown by the tube method as well as on the plate.

TABLE 3
The production of diastase by actinomycetes

NAME OF ORGANISM	TUBE METHOD— HEIGHT IN NUMBER OF MILLIMETERS	PLATE METHOD
<i>A. violaceus-ruber</i>	20	++
<i>A. violaceus-caesari</i>	23	+++
<i>A. viridochromogenus</i>	10	+
<i>A. aureus</i>	22	++
<i>A. albus</i>	22	+++
<i>A. exfoliatus</i>	14	++
<i>A. griseus</i>	31	+++
<i>A. poolensis</i>	13	+
<i>A. albosporus</i>	32	++++
<i>A. lipmanii</i>	32	+++
<i>A. scabies</i>	8	+
<i>A. fradii</i>	25	+++
<i>A. diastaticus</i>	39	++++
<i>A. bobili</i>	24	+++
<i>A. verne</i>	22	++
<i>A. rutgersensis</i>	30	+++
<i>A. asteroides</i>	9	0
120.	20	++
168	42	+++
104	16	+
154.	20	+++
128.	24	+++
215.	24	++
205.	22	

+ indicates zone 1 to 4 mm. wide; ++ 5 to 10 mm.; +++ 10 to 15 mm.; ++++ above 15 mm.

The process of the transformation of starch through the dextrin stages to reducing sugars is very rapid; in some cases the starch, added to the liquid medium to make up a concentration of 1 per cent, disappeared in seven days, and in many cases in fourteen to fifteen days. The source of nitrogen seems to have

an important bearing upon the hydrolysis of starch by Actinomycetes; Krainsky (1914) has already called attention to this fact, showing that asparagine will stimulate greater diastase activity than KNO_3 , NH_4Cl or peptone. The chromogenus types represented in the above table by *A. scabies* and *A. viridochromogenus* have very weak diastatic power. This has already been pointed out before by Krainsky (1914) who stated that the chromogenus species produce very little diastase or none at all.

Four methods were used for the demonstration of cellulose digestion. First the plate method of Kellerman and McBeth described by McBeth (1916); second the method suggested by Krainsky, which consists in placing two pieces of filter paper in Petri dishes, and moistening these with MgNH_4PO_4 solution containing 0.1 per cent of K_2HPO_4 ; third and fourth the addition of filter paper or cellulose suspension to the Czapek's solution without the sucrose as shown above.

The results obtained by the different methods are not concordant. It seems as if some organisms can use the cellulose in one form while others in another. And since all these methods are only poor imitations of natural phenomena and are far from comparing with the natural conditions, under which the organisms live (the digestion of cellulose in the soil is being studied at present), the cellulose decomposition by any one method cannot serve as a positive indication of the ability of a certain organism to attack cellulose. The results are given in table 4.

The cellulose-dissolving power of many Actinomycetes is thus found to be positive by all the methods used. The plate method of Kellerman and McBeth, although well adapted for the study of the growth of the organisms, does not offer any greater advantage than the other methods, since an enzymatic zone was observed for only a few species. The growth of most of the organisms upon this plate was very good, but, as Krainsky (1914) pointed out, this may not be due to the cellulose, but to the agar of the medium. In the growth of the Actinomycetes upon the filter paper, either in the plate or in the solution, free from any other carbohydrate, certain distinctive differences were observed. Certain organisms, such as *A. verne*, *A. exfoliatus*,

and others produced small white colonies right upon the surface of the paper; these separated afterwards and floated through the medium, then falling to the bottom or remaining suspended. Others, such as 120 and *A. violaceus-caesari*, grew upon the paper forming black zones around the colony. Organism 120

TABLE 4
The decomposition of cellulose by actinomycetes

NAME OF ORGANISM	PLATE METHOD (KELLERMAN AND M'BETH)	FILTER PAPER (KRAINSKY)	CELLULOSE SUSPENSION IN SYNTHETIC SOLUTION	PAPER STRIP SYNTHETIC SOLUTION
<i>A. violaceus-ruber</i>	2+	2	3	2
<i>A. violaceus-caesari</i>	1	2	3	2
<i>A. aureus</i>	5	2	0	0
<i>A. albus</i>	3+	5	0	0
<i>A. exfoliatus</i>	3+	1	1	2
<i>A. verne</i>	0		1	3
<i>A. griseus</i>	1	0	1	1
<i>A. poolensis</i>			0	1
<i>A. albosporus</i>			0	0
<i>A. lipmanii</i>		3	—	0
<i>A. scabies</i>	3		—	2
<i>A. fradii</i>	2	4	0	0
<i>A. diastaticus</i>	1		0	0
<i>A. bobili</i>	3	0	0	4
<i>A. virodochromogenus</i>	2	4	3	1
<i>A. reticuli</i>			3	—
120			1	4
104		0	1	1
168	1	1	1	3
128	1	2	0	1
154	2	1	0	2
205	2		0	2
215	4	5	2	2

0 = no growth; 1 = faint; 2 = fair; 3 = good; 4 = excellent growth; + = production of clearing on cellulose plate.

gave the same characteristic growth upon cellulose as that observed by Krainsky (1914) for his *A. melanosporeus* and *A. melanocyclus* with which 120 is doubtless closely associated.

The utilization of cellulose, where this is the only source of carbon, is also demonstrated by the active reduction of nitrates to nitrites and change in reaction, as shown in table 2.

SUMMARY

1. The utilization of different mono-di- and poly-saccharides, alcohols and salts of organic acids as sources of energy for different Actinomycetes was studied.

2. The order of utilization of the different carbon compounds is as follows:

Starch followed by glucose, lactose, maltose, glycerin, sucrose, cellulose and the organic acids.

3. The best growth is usually accompanied by the greatest reduction of the nitrates of the medium to nitrites and commonly by the greater increase of the pH value of the medium.

4. Few Actinomycetes produce invertase, but even those that do not form this enzyme utilize sucrose to some extent.

5. The production of diastase by Actinomycetes is very prominent and characteristic of the whole group, with very few exceptions. The starch is reduced through the dextrin stage to reducing sugars. In only one or two instances does the reduction seem to stop at the erythrodextrin stage.

6. Some Actinomycetes dissolve cellulose readily.

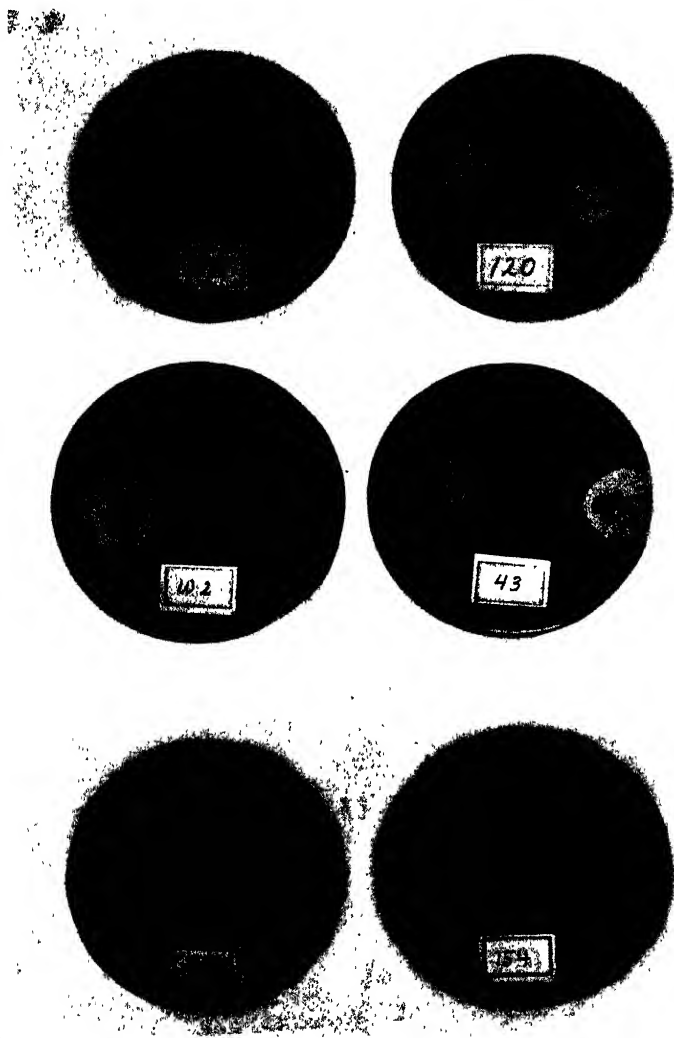
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EXPLANATION OF PLATE 1

Upper photographs (510 and 120), little or no diastase production; the white center indicates the growth of the organisms, while the faint white zone around the growth indicates the diastase production. Middle two photographs (W. 2 and 43), medium diastase production. Lower two photographs (69 and 154), good diastase production. The plates are of regular size (9.25 cm. inside diameter). The cultures were grown for twelve to fourteen days at 25°C., then surface of plate was covered with KI-I solution. The hydrolyzed portion is white, unhydrolyzed blue.



(Waksman. Metabolism of Actinomyces)

A SYSTEMATIC STUDY OF THE PROTEUS GROUP OF BACTERIA

JOHN J. WENNER AND LEO F. RETTGER

From the Sheffield Laboratory of Bacteriology and Hygiene, Yale University

Received for publication March 8, 1919

The term *Proteus* signifies changeability of form, as personified in the Homeric poems in Proteus, "the old man of the sea," who tends the seaflocks of Poseidon and has the gift of endless transformation. The first use of this term in bacteriological nomenclature was made by Hauser (1885) who described under this term three types of organisms which he isolated from putrefied meat. Variations in form and size and in cultural characters were the basis of his classification. Other investigators have since applied the name *Proteus* to specific organisms which they isolated from various sources. Of these organisms some show close similarities and in many instances are identical with the types described by Hauser. Some do not appear, however, to have sufficient common properties to justify inclusion in the *Proteus* group, and attempts to place them here tend to further obscure the poorly defined limits of this group.

The three species which Hauser described under the genus *Proteus* were as follows: *Proteus vulgaris*, which liquefied gelatin and formed zooglea in this medium, and which was very active in its various physiological properties; *Proteus mirabilis* which likewise liquefied gelatin and formed zooglea, but which was less active; and *Proteus zenkeri*, which was unable to liquefy gelatin and which was relatively inactive. The main basis for distinguishing the three species appears to be their action on gelatin.

Babes (1889) isolated an organism from a case of lung gangrene in man which he called *Proteus lethalis*, and another from the

organs of a child which died with symptoms of septicemia, *Proteus septicus*. The same bacterium was later described by Kruse (1896). These organisms appear to be very closely related to one or two of Hauser's types, if indeed they are not identical with them.

Jaeger (1892) isolated a fluorescent organism which, due to some points of similarity with the *Proteus* group, he called *Proteus fluorescens*. It was described as the causative factor in several cases of Weil's disease. It has since been isolated from similar cases by Bar and Renon (1895), by Conradi and Vogt (1901), and by Bruning (1904). While this organism in a general way bears some resemblance to the *Proteus* group, it is so atypical that its inclusion in this division may be seriously questioned. A more natural grouping would place it in the genus *Pseudomonas*.

Fuller and Johnson (1899) describe two spore-forming organisms as *Proteus*. The property of forming proteus-like colonies on gelatin seems to be their sole basis of classification, hence these organisms may be eliminated from further consideration, especially because no other spore-producing bacteria have been referred to the *Proteus* group.

Prior to the work of Hauser an organism was described by Kurth (1883) which on account of its marked resemblance to Hauser's *Proteus zenkeri* deserves mention here. Kurth's *Bacterium zopfii* was isolated from the intestine of fowls, and has since been observed by others on numerous occasions.

The purpose of the present investigation was to determine the exact relation of the *Proteus* group to other groups of organisms, and to point out more clearly than has been done heretofore the specific properties which serve to distinguish the members of what has so generally been termed the "*Proteus* group."

For this purpose 84 strains were obtained from different sources. Of these 58 were procured from other laboratories, and were labelled as follows:

<i>B. proteus-vulgaris</i>	27
<i>B. proteus-mirabilis</i>	8
<i>B. proteus-zenkeri</i>	4
<i>B. proteus</i>	13
<i>B. zopfii</i>	5
<i>B. proteus-viridis</i>	1

Twenty-six different strains were isolated in this laboratory, of which 25 were of the *Proteus vulgaris* or *Proteus mirabilis*, and one of the *B. zopfii* or *Proteus zenkeri* type.

A morphological and cultural study of the organisms of this collection showed that it could be divided into the three following divisions:

Group I comprising *Proteus vulgaris*, *Proteus mirabilis*, and *Bacillus proteus*.

Group II comprising *Proteus zenkeri* and *Bacterium zopfii*.

Group III comprising *Proteus fluorescens*.

The members of group I are Gram negative and very actively motile, and on agar show a peculiar spreading growth. They usually exert proteolytic action on gelatin and in milk, and to some extent attack carbohydrates, and protein material in general. Furthermore, they grow luxuriantly on all of the ordinary media, and are not limited to any specific temperature range.

The two types which comprise group II are distinctly Gram-positive. They possess no proteolytic action and do not attack carbohydrates; neither do they produce a luxuriant spreading growth on moist agar, as do the members of group I. They develop very poorly in liquid media.

The one available strain of group III differed markedly from the organisms of the other two groups. Fluorescent pigment production, together with its other properties so characteristic of the fluorescent group, should naturally place this strain within the genus *Pseudomonas*.

While Hauser at first described the *Proteus* group as being composed of three distinct species, that is *P. vulgaris*, *P. mirabilis*, and *P. zenkeri*, he later thought that the last two species might be only varieties of *Proteus vulgaris*. His latter conclusion seems to have been accepted by most investigators, though little evi-

dence can be found to substantiate it. Kendall (1916) states "that it is now recognized that cultures of *B. proteus* may gradually lose their gelatin-liquefying power after prolonged cultivation, so that a cultural transition from *B. proteus* to *B. zenkeri* may be observed in the laboratory." While we have observed loss of ability to liquefy gelatin in certain strains, we have never noted other changes in *Proteus vulgaris* which would tend to give it the characters of *Proteus zenkeri*.

In the present study the strains of *Proteus* which were labelled *Proteus vulgaris* and *Proteus mirabilis* when received were found to be practically identical in all of their characters. Both liquefied gelatin with the same rapidity. Although in each species variability in proteolytic action was noted in a few instances, no other changes accompanied the partial or complete loss of gelatin-liquefying power, and the strains did not in the least assume the characters of Hauser's *Proteus zenkeri*. Thus it appears that the classification of Hauser holds only in so far as the separation of his species *Proteus zenkeri* from the other two is concerned.

Kruse (1896) and Chester (1909) noted a similarity between Hauser's *Proteus zenkeri* and *Bacillus zopfii* of Kurth (1883). We have found the two to be identical, and hence would classify them as one and the same genus under the generic name of *Zopfius*.

The types which were labelled *Proteus vulgaris*, *Proteus mirabilis* and *Bacillus proteus* have been reduced by us to two species, namely *Proteus vulgaris* and *Proteus mirabilis*, as *B. proteus* is but another name applied to either or both of the others. The *Proteus* group as a whole is sometimes referred to as *Bacillus proteus*; but the use of this name should be discontinued.

In the present investigation the original *Proteus* group of Hauser has been split, therefore, into two distinct genera, namely *Proteus* and *Zopfius*. In the former are included *P. vulgaris* and *P. mirabilis* of Hauser, together with the strains in our collection labelled *Bacillus proteus*, and under the genus *Zopfius*, *Bacterium zopfii* of Kurth and *Proteus zenkeri* of Hauser. The basis for this classification will be brought out further in the data and discussions which follow.

GENUS PROTEUS

This genus may be defined as comprising organisms which in form are small coli-like rods with rounded ends and occurring singly, in pairs or in chains; they are Gram negative, form neither spores nor capsules, and are actively motile by means of peritrichiate flagella. Gelatin is usually liquefied rapidly, though this property may be entirely lost. When inoculated into the condensation fluid of slant agar tubes a rapidly spreading growth is produced over the entire surface of the agar. The strains ferment, with acid and gas production, glucose, levulose, galactose, sucrose and glycerol and occasionally maltose. Alkalinity is usually produced in litmus milk, followed by decoloration of the litmus and digestion of the casein. At times there is slight coagulation or precipitation of casein with subsequent re-solution or digestion.

Organisms of this genus are widely distributed in nature, and have been isolated from numerous sources. Their presence in soil appears to depend largely upon recent contamination with animal excreta or putrefactive organic matter of animal origin. Cantu (1911) was able to isolate organisms of this genus from 23 out of 52 samples of garden soil.

Members of this genus are often present in stagnant pools, sluggish streams and other contaminated waters. We have obtained them from stagnant pools, aquaria and street washings. Ward (1899) isolated several strains from the Thames River, and Jordan (1903) from the waters of the upper Mississippi. Horowitz (1916) made several isolations from snow water.

Proteus organisms may be said to be present in practically all sewage, for here there is a constant source of contamination and a favorable medium for development.

The presence of this genus in the intestinal tract of man is by many authorities regarded as an indication of intestinal trouble or some other pathological condition. Ford (1901) claims to have isolated it from various parts of the intestine, but as some of his organisms were fermenters of lactose, there is some doubt as to whether all were *Proteus*. Stewart (1917) be-

believes that *Proteus* members found in war wounds are of non-fecal origin. In the examination of several thousand samples of feces from dysentery convalescents he found this genus to be a very uncommon inhabitant of the colon of man. It may be obtained from the intestinal tract of lower animals, as for example guinea pigs. Jensen (1903) observed this genus to be present in large numbers in calves affected with a form of dysentery. Its presence in similar conditions in man and animals may in part account for its wide distribution in nature.

The most favorable habitat of the genus *Proteus* is decomposing organic matter of animal origin. In such material it is almost invariably present. Cantu (1911) was able at will to isolate it from putrefied meat, as have many other investigators. Wyss (1898) obtained a strain of *Proteus* from dead fish, and Shrank (1888) from spoiled eggs. Isolations have been made also from human cadavers, where this organism was found in large numbers by Hauser (1885), Hofmeister (1893), Haegler (1892) and Kuhn (1891). We were able to obtain it from meat which had been allowed to undergo decomposition, and from the partly decomposed bodies of dead rabbits and guinea-pigs.

Method of isolation

Until quite recently the usual gelatin and agar plate methods of isolation have been employed for this group. As these were very faulty for this type of bacteria, many efforts resulted in failure. The newer methods have rendered valuable service, however. In the present work the procedure of Cantu (1911) was at first adopted. Gelatin tubes are inoculated directly with the material in question. After incubation at 20°C. for several days transfers are made from tubes in which liquefaction has taken place to the condensation fluid of new slant agar tubes. If *Proteus* organisms are present a rapidly spreading growth occurs in twelve to twenty-four hours at 30° to 37°C. This growth is quite characteristic and usually spreads over the entire surface. From the uppermost portion of the surface growth inoculations are made in the condensation fluid of a second agar tube, and the process repeated until a pure culture is obtained.

It soon became apparent in the present investigation that the materials for study could be inoculated directly into the condensation water of the sloped agar tube, and the period in which isolation is effected very much shortened. This modification in no way detracts from the merits of the Cantu procedure. As a rule very little effort is required to effect complete isolation of the *Proteus* genus, owing to its peculiar property of overspreading agar rapidly and leaving associated organisms behind in the condensation fluid. Fresh agar is necessary, however, and the results are greatly facilitated by washing the agar surface with the condensation water just before inoculation.

General characters of the Genus Proteus

The salient features of this genus have already been defined. The following is an elaboration of the different characters, in so far as Journal space will permit.¹

The individual cells are usually short Coli-like rods with rounded ends, varying in dimensions from 0.4 to 0.6 μ by 1.2 to 2.5 μ , though occasionally much longer cells are seen. The rods may be grouped singly, in pairs or in short chains. They are actively motile, possessing peritrichous flagella. The unstained cells appear homogeneous in structure. Neither spores nor capsules have been observed. All strains are at all times Gram-negative. Young cultures are readily stained with methylene blue, fuchsin and other common basic dyes.

Members of the *Proteus* genus grow luxuriantly on the usual solid and liquid laboratory media. They are capable of growing within a wide range of temperature, and within reasonable limits development is not materially affected by change in hydrogen ion concentration.

Various ranges of temperature have been reported as most favorable. Hauser (1885) gives 20° to 34°C. as the optimum. Kendall (1916) places it at about 25°. Berthelot (1914), Cantu (1911), and Glenn (1911) grew the organisms successfully at 37°. Levy

¹For more complete descriptions and discussions the reader is referred to the doctorate thesis (J. J. Wenner) in the Yale University Library.

(1894) showed that the group develops slowly at a temperature as low as 0° and as high as 43° to 45°C. We have invariably obtained maximum growth at 34° to 37°. Good growth was obtained also at 20°, though longer incubation was required, as shown for example in glucose broth culture in which maximum acidity was attained in twenty-four hours at 37°, as against forty-eight hours at 20°, and maximum gas production in twenty-four hours at 37°, as against one hundred and twenty hours at 20°C.

Growth on plain agar

The most characteristic growth of *Proteus* is obtained on slant agar. This was pointed out by Cantu (1911) when he showed that inoculation in the condensation fluid of fresh sloped agar resulted in a uniform growth over the entire surface. This growth may be homogeneous, or of a more or less peculiarly modeled character. It is of a butyrous consistency. If the surface of the inoculated agar is dry a streak inoculation results in a pronounced growth which spreads very irregularly, with a more or less lacerated margin. The extent of the spreading depends on the amount of moisture on the agar.

Colony growth on plate agar may be at times characteristic, that is, of ameboid appearance, or in the form of large colonies which are more or less rosette-like, with very irregular borders. Again, the colonies may be small and with entire margin, resembling those of *B. coli*.

Action on gelatin

Much interest has centered around the property of gelatin liquefaction of this genus. Hauser in his original work laid special emphasis on it and used it as the chief basis for his distinction of types. Since then marked variations in individual strains have been observed by different investigators. Smith (1894) was able by selection to transform a liquefying *Proteus vulgaris* into a non-liquefying strain. Herter and Broeck (1911) showed that a liquefying strain of *Proteus vulgaris* which had

lost its liquefying properties, but remained typical in other respects, could have the lost function restored by passage through a mouse.

Of the 73 strains studied in this investigation, 3 lost the property of liquefying gelatin while in other ways they remained typical. Two of these organisms were old laboratory strains labeled *Proteus vulgaris*; the third was isolated from putrefying meat. We were unable to restore the liquefying function by a single passage of one of these strains through a white rat.

On gelatin plates of *Proteus* small colonies are noticeable in eighteen to twenty-four hours. They show an entire margin at first, but as they increase in size irregular spreading may occur. Liquefaction soon takes place and the colonies assume a dew-drop appearance. Radiating filaments extend from the liquefied zone into the surrounding gelatin. The colonies increase in size until the entire plate is liquefied. Hauser employed 5 per cent gelatin, and describes the occurrence of wandering ameboid colonies, that is, irregular masses of cells which constantly underwent changes in form and position, and sometimes separated from the mother colony. In order to obtain colonies that are at all characteristic gelatin of rather soft consistency is required. On the usual 10 per cent gelatin the colonies are often entire and without distinguishing marks.

In gelatin stab cultures liquefaction begins at the surface, soon becomes stratiform and eventually involves the entire tube. The rate depends on the temperature and an abundance of free oxygen. Liquefaction may be completely inhibited by a layer of oil over the surface of the gelatin. The oxygen is essential in the production of the proteolytic enzyme.

Growth in bouillion

Marked turbidity is rapidly produced, reaching its maximum in from three to five days at 30 to 37°C. Young cultures usually show no surface film, while older tubes gradually develop a thin brittle pellicle which is easily broken up. As broth cultures present few if any features which are characteristic and of special

interest no further comments are necessary. Nitrite is formed in nitrate broth.

Action of the genus Proteus in milk

As a rule vigorous development occurs in milk, and a marked change may be brought about in the appearance of this medium in twenty-four to thirty-six hours at 37°C., the litmus being reduced and coagulation or digestion of the casein taking place. The rate of transformation varies with different strains, some of them completely digesting the casein in three to five days. On the other hand, other strains appear to have lost this proteolytic power completely. The usual change observed in this study of 73 strains was an initial alkalinity which gradually became more intense and was followed by decolorization of the litmus and digestion of the casein. Some strains (3) showed slight acid production at first. Casein was digested by 69 strains.

The ability of organisms to digest casein was demonstrated definitely by growing them in a medium containing, besides 0.5 per cent meat extract and 0.5 per cent sodium chloride, 0.2 per cent of purified casein, and observing the loss of the protein by means of the biuret method of Vernon (1903), or by precipitation with acetic acid.

Action on carbohydrates, glycerol, etc.

Fermentation is limited to glucose, levulose, galactose, sucrose, maltose and glycerol. The glucose, levulose, galactose and glycerol were attacked more or less uniformly by all strains, sucrose readily by some and slowly by others, and maltose only by some of the strains. Fermentation in all cases comprises both acid and gas production. The medium employed in the fermentation experiments was plain sugar-free broth to which 1 per cent of the carbohydrate in question was added. Other agents used were lactose, inulin, dulcitol, mannitol, sorbitol, salicin, raffinose, arabinose, adonitol, dextrin and starch. The results with these were negative.

Glucose

This is one of the most favorable sources of energy for the organisms of the *Proteus* genus. Its presence in a medium considerably hastens growth. From 25 to 30 per cent of gas, and from 2.5 to 3 per cent of acid in terms of N/20, with phenolphthalein as an indicator, are produced. These results agree with those of other investigators.

Sucrose

Smith (1893) was the first to show that the action of this group on sucrose was practically the same as on glucose. Similar results have been obtained since by other investigators, though Glenn (1911) found several indifferent strains among his stock cultures, and Horowitz (1916) reports a positive reaction in only 7 out of a total of 24 strains.

In the present investigation a variation in the action of *Proteus* on this sugar was noted, some strains producing the maximum amounts of acid and gas in twenty-four to seventy-two hours, while others required twelve to fifteen days. The delayed action of the latter (8 or 9 days) was at first overlooked, but it was observed that when the period of delayed action was passed the fermentation was as pronounced as with the strains which attacked the sucrose immediately. Of the 73 strains studied, 25 showed an immediate, and 48 a delayed action. In correlating these results with the action on other carbohydrates, it soon became apparent that the strains which fermented sucrose readily also fermented maltose, while those which showed delayed action on sucrose did not attack the maltose.

Maltose

Maltose appears to be the only carbohydrate that is of any value as a means of subdividing the *Proteus* group. Berthelot (1914) noted a variation in the action of different strains on this sugar. Horowitz (1916) found that 23 out of 24 strains fermented it with the production of acid and gas; and Stewart (1917) observed 2 out of 29 having this property. Of the 73 strains in the present collection, 25 showed distinct acid and gas production. No

delayed action on the sugar could be detected, as in the case of sucrose.

Galactose, levulose and glycerol

While these agents are fermented by this group the action is not so marked and does not occur as readily as with the sugars just mentioned. The amount of gas produced may vary from a mere bubble to 20 per cent, and the acid from 1 to 2 cc.

Lactose

Conflicting results have been reported. While most investigators have claimed that lactose is not attacked by the *Proteus* group, others have observed fermentation with acid and gas production. In the light of our own experiments these conflicting results may be explained by the presence of an available carbohydrate as an impurity in the lactose. When absolutely pure lactose was employed no fermentation could be detected under either aerobic or anaerobic conditions.

Growth on potato

On cooked potato prepared in the usual way very luxuriant growth is produced. It appears within twenty-four hours along the line of inoculation and gradually spreads over the surface irregularly. It is of a butyrous consistency and of a dirty brown color which quickly diffuses through the potato. A characteristic fish brine odor is produced in this medium.

Browning of lead acetate medium

All of the 73 strains of *Proteus* used in this investigation caused a distinct browning of a medium consisting of 0.5 per cent nitrate agar, 0.05 to 0.1 per cent lead acetate, and 0.2 per cent glucose.

Hemolytic action

This genus is unable to hemolyze red blood cells. Different strains were tested both in suspensions of washed erythrocytes and on plates of sterile blood agar.

Growth in synthetic media

Development in Uschinsky and similar synthetic media is limited. It becomes more marked, however, when glucose is substituted for glycerol in the medium. In the phthalate medium of Clark and Lubs (1917) growth is likewise limited.

Chromogenesis

With the exception of a few investigators (Ward, 1899, and Jordan, 1903) the *Proteus* group is considered as non-pigment producing. In the present work no color production was noted in any of the media except the brownish growth on potato and the gradual browning of the potato itself.

Changes in hydrogen ion concentration

In plain bouillon prepared from Liebig's beef extract and Witte's peptone no change in titratable acidity was noted, while hydrogen ion determination by the newer colorimetric method showed slight alkali production. In plain bouillon containing an available carbohydrate sufficient acid is produced to bring the H ion concentration to about 5 on the colorimetric scale. Similar results were obtained in the special peptone medium of Clark and Lubs (1917). Little acid production occurs, however, in their phthalate medium owing to the limited growth of the organisms.

Indol production

Indol production by this genus has been pointed out by many investigators. Variations in this property have been noticed also. Steensma (1906) studied several strains which failed to produce indol. Van Loghem and Van Loghem-Pouw (1912) made two subdivisions out of the strains under observation, namely *B. proteus-anindologenes* and *B. proteus-indologenes*. Berthelot (1914) found that 24 out of a total of 61 strains formed indol; Horowitz (1916), 7 out of 24; and Stewart (1917) 1 out of his collection of 29.

In the present work results were obtained which varied with the methods employed. Dunham's solution, sugar-free broth, and a 1 per cent solution of predigested casein were used. Both the Salkowski and the Ehrlich aldehyde method were employed. Of the 73 strains all gave a positive reaction with the sulphuric acid and nitrite test of Salkowski, while 46 gave a reddish color on the addition of the acid alone. With the Ehrlich method 33 of the 73 strains gave a strongly positive, 36 a slightly positive and 4 a negative reaction. These variations were obtained in each of the 3 media.

Hydrogen sulphide and mercaptan

All of the strains formed hydrogen sulphide in appreciable amounts. On the other hand, little if any mercaptan could be detected. Mercaptan production has been the subject of investigation on previous occasions. It has been assumed by many that this is a common product of *Proteus*, because this genus is so constantly present in organic matter undergoing putrefactive decomposition, though it is not itself a strictly putrefactive organism. Rettger (1906) found no mercaptan in anaerobic cultures of *Proteus vulgaris* in egg-meat mixture. Herter and Broeck (1911) also were unable to detect it in plain bouillon cultures, even when cystin was added. Ward (1916) claims, however, that he obtained marked mercaptan production with 4 different strains which he grew in plain bouillon.

Nine strains were tested for the property of mercaptan production by the method formerly employed by Rettger, and involving the use of isatin-sulphuric acid and of mercuric cyanide. In some instances a slight change in the color of the test solutions could be detected, but as control flasks gave a similar change in color, little, if indeed any, mercaptan was present in the culture flasks. Contamination of such flasks with a putrefactive anaerobe, however, soon resulted in abundant mercaptan production.

Putrefaction

The experiments of Hauser (1885), Emmerling (1896) and others, demonstrating putrefactive changes in what appeared

to be pure cultures of *Proteus* organisms, as well as the frequent assertions that members of the *Proteus* group are always present in organic matter that is undergoing putrefaction, has led to the assumption that this group has distinct putrefactive properties. Rettger and Newell (1912) have shown more recently that no decomposition of protein material takes place under anaerobic conditions when pure cultures of *Proteus* are used. Similar experiments were conducted in the present investigation, and the results of Rettger and Newell corroborated. No changes in the character of protein material could be brought about by pure cultures of *Proteus vulgaris* in the absence of atmospheric oxygen, whether in milk, egg-meat mixture, or other protein-containing medium. There was no reduction in the volume of the solid matter in the egg-meat medium, nor could any of the foul smelling products of putrefaction be detected. Furthermore, there was very little, if indeed any mercaptan present in the medium. Under aerobic conditions, however, the ordinary non-putrefactive products of protein decomposition are produced.

Agglutination

Several attempts have been made in the past to employ agglutination as a basis for subdividing the *Proteus* group. Cantu (1911) showed that the blood serum of animals which had been injected with heated suspensions of these organisms had agglutinating properties which, barring some exceptions, were specific for the strains injected. He concluded that this method can not be employed for subdividing different strains. Van Loghem and Van Loghem-Pouw (1912) claimed that indol-producing strains could be distinguished from those which do not form indol, by their agglutination properties. Horowitz (1916) obtained cross agglutinations among homologous strains, and thereby was able to split the *Proteus* group into 5 subdivisions, the members of each having specific properties, as regarded indol production and carbohydrate fermentation.

In the present work several rabbits were immunized against specific strains of *Proteus vulgaris* and *Proteus mirabilis*. Killed

suspensions were injected at first, followed by at least one or two suspensions of living organisms grown on slant agar and washed off with saline solution. After each injection the animals showed some loss in weight which was very soon regained. At the site of inoculation a large abscess was formed which disappeared only after several months. The production of agglutinins could be demonstrated very soon after the first injection. After the last injection agglutination in as high as 1:100,000 dilution took place.

The different strains of *Proteus* were tested by the macroscopic method in dilutions of 1:50, 1:100, 1:500, 1:1000, and 1:5000. Seven different sera were prepared with as many strains of *Proteus*. Table 1 shows the number of strains agglutinated by each serum. With one exception, all of the sera agglutinated other strains besides those employed in their preparation. Some strains were agglutinated by more than one serum. Nineteen of the strains used in the agglutination tests failed to be agglutinated by any of the sera. It would appear, on the whole, that the *Proteus* group is more or less heterogeneous, like the *Streptococcus* and *B. dysenteriae* group. While the agglutination method may be of some value, in identifying members of the *Proteus* group, negative results do not necessarily exclude an organism from this group.

Pathogenicity

The occurrence of the genus *Proteus*, either in pure culture or in association with other organisms, in pathological conditions, has been reported by various investigators. Foa and Bonome (1889) isolated it from a case of volvulus, Schnitzler (1890) and Krogus (1890) from cases of cystitis, Flexner (1893) from a patient having peritonitis, and Reed (1894) in croupous pneumonia, associated with a pneumococcus. Booker (1897) and Metchnikoff (1909) made *Proteus* isolations from cases of diarrhea in children, Vincent (1909) from typhoid fever patients, and Horowitz (1916) from persons suffering with gastro-enteritis. Larson and Bell (1915) recovered *Proteus* organisms from a

laparotomy wound, infected eye and finger, from the heart's blood of a fatal case of peritonitis, and from one of gangrene of the lung. Ward (1916) obtained it from supposed diphtheria subjects and from typical cases of atrophic rhinitis.

Dudgeon, Gardner and Bantree (1915) found typical *Proteus* in 5 per cent and atypical *Proteus* in 2 per cent of a total of 100 cases of war wounds. Goadby (1916) encountered *Proteus* in 47 per cent of the 200 wounds studied bacteriologically. Distaso

TABLE 1

Number of strains agglutinated by each serum or combination of sera

NUMBER OF STRAINS	SERA*						
	A	B	C	D	E	F	G
1	+	0	0	0	0	0	0
1	+	+	0	0	0	0	0
1	0	0	0	0	0	+	0
8	+	+	0	0	+	0	0
3	0	+	0	+	0	0	0
1	0	+	+	+	0	+	0
1	0	0	+	+	0	0	0
23	0	0	+	+	0	+	0
11	0	0	0	+	0	0	0
3	0	0	0	+	+	0	0
1	0	0	0	0	0	0	+
19	0	0	0	0	0	0	0
Total, 73	10	13	25	42	11	25	1

* Sera A, B, C, D, E and F were prepared with stock strains of which the first five were labeled *Proteus vulgaris* Hauser and the last *Proteus mirabilis* Hauser. Serum G was prepared with a strain isolated by us from putrefied meat.

(1916) found coliform organisms including *Proteus* predominating in the first stages of wound infection. He suggests the use of *Proteus* vaccine along with others in the treatment of war wounds. Stewart (1917) isolated 29 strains of the *Proteus* genus from infected war wounds, or a case rate of 24 per cent.

While this genus is ordinarily regarded as non-pathogenic, there is ample evidence to show that it may assume a pathogenic rôle, and thus occupy a position analogous to the pyogenic micrococci. The pathogenicity varies in experimental animals,

some strains causing death in 16 to 24 hours, while others cause no apparent ill effects. For example, Kühnau (1897) found that strains from several cases of diphtheria, and Larson and Bell (1915) that some strains of *Proteus* obtained from human lesions, were decidedly pathogenic for rabbits, guinea-pigs and rats. In general the virulence of a strain is shown by the production of local pathological conditions or by symptoms of intoxication.

In the present investigation both virulent and non-virulent strains were met with. One of the most pathogenic was an old stock culture of unknown origin, this indicating that virulence may be maintained indefinitely.

Hauser, in his original work on the *Proteus* group, found that bouillon and gelatin cultures were toxic and produced fatal results when injected into animals. Other investigators have obtained similar results. The nature of the toxicity is not known, although the effects are apparently those of real toxemia.

The toxicity of several strains of the *Proteus* genus was demonstrated by the writers by injecting 2 cc. of saline suspensions from 24-hour agar cultures. Subcutaneous injections in rabbits produced abscesses and inflammatory conditions which lasted several months, usually accompanied by loss of weight, weakness and lessened appetite. In white rats the results varied with the strains, some causing symptoms of toxemia and killing the animals in eighteen to twenty-four hours, when injected by the subcutaneous route. Others caused no apparent ill effects even when the injections were intraperitoneal. One strain caused the formation of an abscess in one rabbit, and definite symptoms of toxemia and death in another. In the fatal cases the organisms could be isolated from the blood and internal organs. Killed suspensions when injected into rabbits caused definite lesions at the site of inoculation.

Classification of species

Since Hauser's classification several investigators have attempted to group the various strains of the genus *Proteus* on properties other than gelatin liquefaction. Ford (1901) defines

the *Proteus* group as consisting of alkali-producing non-chromogenic, non-sporing bacilli capable of liquefying gelatin, casein and blood serum. He made a further division, on the basis of motility and carbohydrate fermentation, into six varieties, two of which fermented lactose.

In his study of bacteria found in river water, Jordan (1903) divided the *Proteus* group into two subdivisions, namely the *Proteus vulgaris* type and *Proteus* varieties. The first of these he described as always fermenting glucose and sucrose, with gas production, but never lactose; liquefying gelatin, casein and blood serum, and curdling milk, with acid production. The second subdivision differed from the first mainly in its proteolytic action. Cantu (1911) in a study of 184 strains isolated from various sources was unable to subdivide them. Van Loghem and Van Loghem-Pouw (1912) were able to divide a series of strains obtained mostly from intestinal contents into two groups on the basis of their indol-producing function. The strains belonging to one or the other group were similar in their agglutinating properties. Horowitz (1916) divided 24 strains into 5 subgroups, on the basis of agglutination. Stewart (1917) found 27 strains isolated from war wounds to differ in their action on maltose and litmus, and in their motility and indol production.

The present investigation has shown that attempts of others to divide the *Proteus* group into two or more subdivisions are unsound. The classification of Hauser on the basis of gelatin liquefaction is of little value, since this property is too irregular and inconsistent. In their agglutination power the members of the *Proteus* genus are heterogeneous in character, so that no distinct separation into species is possible on this basis. Indol production is also very unsatisfactory as a distinguishing character. The only property which appears to us to be of value in making subdivisions of the genus *Proteus* is that of carbohydrate fermentation. Several investigators have noted a difference in the action of individual strains on maltose. Of the 73 strains employed by us 25 fermented this sugar, while the remaining 48 failed to do so. A definite correlation existed between the property of attacking maltose and

the rapidity with which sucrose was fermented with gas production. All of the strains which fermented maltose, with both acid and gas production, also fermented sucrose readily, while all of those which failed to attack maltose showed a delayed action on sucrose and brought about visible acid and gas production only after the expiration of eight to nine days.

While no other property could be correlated with this action on the sugar, it lends itself as a definite basis for dividing the *Proteus* genus into two species, the one fermenting maltose with acid and gas production, and the other being unable to attack this disaccharide. For the former the name *Proteus vulgaris* may be retained, while for the other *Proteus mirabilis* is here suggested. By retaining these names the nomenclature would be simplified. The differentiating characters of Hauser must be set aside, however, in order to avoid confusion.

GENUS ZOPFIUS

Under this genus the types formerly known as *B. zopfii* and *Proteus zenkeri* will be described. Very few strains of these organisms are kept in stock, as only 4 strains of *Proteus zenkeri* and 5 strains of *B. zopfii* could be obtained by a canvass of 40 bacteriological laboratories. To these 9 strains one was added which we were able to isolate from putrefied meat. All of these strains were practically identical.

The individual cells are rod-shaped, usually about 0.8μ by 3.5μ in size, have somewhat rounded ends, and in young cultures occur in long evenly-curved chains. They stain well and are Gram-positive. The organisms are motile, having peritrichous flagella, but do not form spores or capsules. They are facultative anaerobes and grow well on the surface or directly beneath the surface of agar and gelatin. In gelatin stab tubes an arborescent growth results which is most luxuriant at the top of the stab. In plain bouillon growth is slow and moderate, while in litmus milk it is very scant and produces no visible change. Gelatin is not liquefied, and none of the carbohydrates are attacked. On potato the growth is moderate with subsequent darkening of

the medium. The most favorable temperature for this genus is about 25°C. Good growth also occurs at 20° and at 30°, while at 37°C. the growth is very poor. No distinguishable odor was noted on any of the cultures. Hydrogen sulfide was not produced and growth in egg-meat medium was poor, resulting in no visible changes. On slant-agar and in agar and gelatin plates a more or less characteristic spider web growth often develops, but inoculations in the condensation water of slant agar do not cause a spreading over the surface. A division of the various strains into species did not seem possible on account of the few differentiating properties of these organisms.

SUMMARY AND CONCLUSIONS

The *Proteus* group has been known to include various types of organisms some of which have few common properties.

The types *Proteus vulgaris* Hauser, *Proteus mirabilis* Hauser, and *B. proteus* are, with a few exceptions, identical. The genus *Proteus* should be limited to organisms of this group.

Proteus zenkeri is identical with *B. zopfii* and therefore should not be grouped with the *Proteus* genus but rather with *B. zopfii*, the organisms of this type forming a genus to be known as *Zopfius*.

The organism *Proteus fluorescens* Jaeger does not resemble the *Proteus* genus, but rather the fluorescent group (genus *Pseudomonas*), and should not be known by the name *Proteus*.

The *Proteus* genus comprises a large group of organisms which can be subdivided on the basis of their action on maltose into two distinct species. For the species fermenting this sugar the name *Proteus vulgaris* is suggested, and for the species failing to attack it the name *Proteus mirabilis*. The genus cannot be subdivided satisfactorily on the basis of proteolytic action, indol production, or agglutinating properties.

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ON THE BACTERIOLOGY OF DYSENTERY IN NORWAY

TH. THJØTTA

From Dr. med. F. G. Gades Pathological Institute, Bergen, Norway; Director: Dr med. M. Haaland

Received for publication March 7, 1919

After the first description of the dysentery bacillus by Shiga and the subsequent detection of the so-called mannit-fermenting strains (pseudo-dysentery bacilli, atoxic dysentery bacilli) by Flexner, Strong and Hiss and Russell the knowledge of these bacilli remained practically unaltered for a rather long time. It is only the last few years that have brought some new development to the study of bacillary dysentery.

Of very great importance in this new development is the description by Sonne in Demark of a hitherto unknown type of the dysentery bacillus. This type, which Sonne calls group III, had been seen by some authors before him (Baerthlein, Losener and others). But these authors had considered the microbe as an inagglutinable type of the Flexner bacillus and had not taken any interest in the study of it. Sonne, however, showed that this microbe serologically (by agglutination) was very specific, and absolutely different from the other dysentery bacilli. He further showed, that this microbe was the main cause of dysentery in the material studied by him in Copenhagen.

After Sonne the microbe was isolated in France by D'Herelle who did not know of the description by Sonne. Further the microbe has been found by the author of this paper in a quantity of cases of dysentery in Norway, and lastly by Oehnell in Sweden.

¹Extract from a larger work "Om Dysenteri i Norge" (On Dysentery in Norway), *Medicinsk Revue*, Bergen, 1917, and from a smaller one "Yderligere bidrag til kjendskapet til dysenteribaciller av gruppe III" (Further contributions to the knowledge of dysentery bacilli of group III), *Medicinske Revue*, juli, 1918.

Another very important result of the study of dysentery during the last years is the statement, that the old types Y, Flexner and Strong cannot any longer be considered as different types. They must be united into one main group of bacilli, that show some individual variation in their fermenting of sugar broth. This statement was first made by Sonne, who arranged those of his types, that corresponded to the old mannit-fermenting types, into two serologically slightly different groups. He considers these groups as closely connected and is of the opinion that they will probably soon be united into one main group. He names them atoxic groups I and II.

Many other authors (Arnheim, Hehewerth, Chick, Pribram and others) are of the same opinion as Sonne and even claim the serological identity of all the mannit-fermenters, or only find small individual variations insufficient to form a just basis of grouping. They also find the cultural characters inadequate for dividing the bacilli into well characterized and different types. It must, however, be mentioned that all these authors except Sonne deal only with the types Y, Flexner and Strong, not with Sonne's group III. Thus it is at the time most convenient to group the mannit-fermenting dysentery bacilli into two main groups, one containing the old types Y, Flexner and Strong, the other group III of Sonne.

The material for this investigation has been stools from patients suffering from typical dysentery or acute diarrhoea, all from Bergen, or neighboring towns or rural districts on the west coast of Norway. Sixty-five strains of dysentery bacilli have been isolated. About 50 of these have been thoroughly studied as to their serological and cultural characters, while the remaining 15 only have been put through the tests necessary to state their identity and classify them in the right group.

The technique of the examination has been to plate out on litmus-lactose-agar shreds of mucus from the stools, which have been washed several times in saline solution. The blue colonies on the plates are tested with highly agglutinating sera from rabbits, immunized with the various strains belonging to the mannit-fermenters as well as with a strain of the non-man-

nit-fermenting type of Shiga. After this the colonies are cultivated and tested as to their fermentation of sugar (mannit, maltose, glucose and sucrose), and the non-motility of the bacilli is established in a young culture in beef broth.

I. SEROLOGICAL CHARACTERS

1. *Agglutination*

The strains first isolated were tested with sera partly from Germany, partly from Denmark. After this the strains were inoculated into rabbits, and there were produced several sera for our Norwegian strains. As the Danish mode of grouping has been used in the classifying of the strains, this will be retained here as well, although it will be shown, that the Danish groups I and II in reality are so closely connected, that they may be considered as one group.

The sera used fall into three different groups:

A. Serum produced with a Shiga strain.

B. Four sera produced with two strains belonging to each of Sonne's groups I and II.

C. Two sera produced with strains belonging to Sonne's group III. With these sera cross agglutination with homologous and heterologous strains has been carried out as shown in table 1. The results have been obtained by naked eye examination after two hours in the incubator at 37°C. As antigen is used an emulsion of living bacilli in saline solution after twenty-four hours of growth on agar.

From table 1 it will be seen that the strains isolated in this material fall into two sharply divided groups. The first one is named B, the second C. The letter A is reserved for the Shiga strain. These letters are not proposed as group names, but used here to avoid confusion with the Danish groups I, II and III. From table 1 it will further be seen, that our group B includes specimens of strains from the Danish group I as well as from group II, while our group C corresponds to the Danish group III.

The groups A, B and C differ distinctly from each other, but so that there is a very slight connection between A and B, and no

TABLE 1

Agglutination in immune sera from rabbits (the figures signify dilution of serum)

TESTED WITH		SERUM FROM RABBITS IMMUNIZED AGAINST				
Groups	Strains	A	B		C	
		Dys. Shiga	Danish group I	Danish group II	Group III	
		Titer 1: 1800	O 38 Titer 1: 6400	F 31 Titer 1: 3200	F 79 Titer 1: 400	
A	Dys. Shiga	1600	400	400	0	
B	Danish group I	O. 36	100	640	3200	0
		F. 11	100	3200	1600	0
		F. 13	100	3200	3200	0
		F. 16	100	3200	3200	0
		F. 51	100	3200	1600	0
		F. 52	100	6400	1600	0
		F. 55	100	3200	1600	0
		F. 60	200	6400	3200	0
		F. 61	100	6400	3200	0
		F. F2	100	6400	3200	0
		F. F3	100	6400	1600	0
		F. F5	100	3200	3200	0
		F. F8	100	3200	3200	0
	F. 90	400	3200	3200	0	
	F. 10F	100	6400	1600	0	
	Danish group II	F. 29	200	800	3200	0
		F. 30	200	1600	3200	0
		F. 31	200	1600	3200	0
		F. 5F	200	1600	1600	0
		F. 92	400	200	3200	0
F. 99		0	200	3200	0	
F. 103		100	400	3200	0	
Group III	F. 108	?	640	6400	0	
	F. 109	?	640	6400	0	
	F. 40	0	0	0	400	
	F. 41	0	0	0	400	
	F. F1	0	0	0	400	
	F. F6	0	0	0	800	
	F. FF	0	0	0	400	
	F. F9	0	0	0	400	
	F. 86	0	0	0	400	
	F. 9F	0	0	0	400	
	I	0	0	0	400	
	II	0	0	0	400	
	III	0	0	0	400	
IV	0	0	0	400		
V	0	0	0	400		
VI	0	0	0	400		
VII	0	0	0	400		
IX	0	0	0	400		

connection whatever between these groups and C. As to the relationship between the Danish groups I and II, the table shows that none of the strains in group I are inagglutinable in the sera from group II and vice versa. The strains of group II, however, show a lower agglutinability in the sera from group I than do the homologous strains of group I. On the other hand, the strains from group I are agglutinated to the same titre by serum from group II as the strains from this group themselves.

Consequently there is some difference in the results of the agglutination between the Danish groups I and II. But this difference does not seem large enough to justify their separation into different groups. We have therefore considered it convenient to place them in one main group.

As to the group C (Danish group III) the table will show the absolutely isolated position of this microbe in the scheme of dysentery bacilli. It will also show the low titre of the agglutination of the strains, belonging to this group compared to that of the other strains. Further the agglutination itself is remarkable in this group. There are never to be observed coarse flocculi in the emulsion here such as are seen in the other groups, but always rather fine flocks.

Out of 65 strains of bacilli isolated in this material 40 belonged to group B, 25 to group C. This is not to be explained on the ground that group C consists of rarer microbes than group B. But while the latter group B as a rule gives rise to typical dysentery and attracts notice and therefore naturally contributes largely to this material, the members of group C often cause only a mild diarrhoea, that is not thought serious enough to be treated by a physician and accordingly is not examined.

The examination of agglutination reactions in the sera of dysentery patients cannot be said to be of any great importance in the diagnosis of the disease as the stools render a better material for the examination at an earlier period in the disease than the serum.

Twenty-two different sera from patients have come to examination. Of these 18 were suffering from dysentery at the

time of examination, while 4 had passed through the disease earlier.

TABLE 2

Agglutination in sera from dysentery patients (the figures signify dilution of serum.)

SERUM FROM CASE NUMBER	CASE BELONGS TO GROUP		DAYS AFTER BE- GINNING OF DISEASE	AGGLUTINATION WITH MICROBES FROM			
				Own strain	Group B		Group C
					Danish group I	Danish group II	Danish group III
52	B	Danish Group I	7	800	200		0
			28	200			
60			3	40	40	40	0
62			10	200	200	200	0
75			14	640	640	40	0
78			10	80	80	40	0
			18	320	320	40	0
90			6		80	160	0
			15	320	320	320	0
99		Danish group II	7		160	160	0
			16	200	200	200	0
			45	320	200	200	0
41	C	Danish group III	5	20	10	10	20
			9	40	10	10	40
71			10	0	0	0	0
86			10	40			40
I			10	40			Not tried
II			10	20			Not tried
III			10	80			Not tried
IV			10	40			Not tried
V			10	40			40
VI			10	80			80
VII			10	10			10
IX			10	40			40

As shown in table 2 all the patients suffering from infections due to group B gave positive agglutination tests at the end of the first week of the disease and later on. The titre did not seem to increase much in the course of disease. Some of the sera only

gave a positive reaction with the most closely connected strain, a fact that shows the necessity of testing the sera from dysentery patients with several strains before calling the test negative.

Eleven sera from patients suffering from infections with strains belonging to group C have been examined. One of these sera showed an absolute negative reaction, while the others gave positive tests in a dilution of serum from 1:10 to 1:80. The same fine flocculation was seen here as described above.

Tested in normal human sera the strains of group B as a rule showed agglutination up to the dilution 1:40, while group C never showed the slightest sign of agglutination in the same sera. It is therefore obvious that even a low titre of agglutination of group C in a certain serum renders it very likely that the patient, from whom the serum is derived, is suffering from an infection due to a member of this group. On the other hand the titre of a member of group B must be higher than 1:40 to allow any conclusions as to infection.

The titre 1:40 is a good deal lower than that which most other authors have found. Thus Sonne found titres in normal sera up to 1:250, Loewenthal up to 1:100, while Frankel only reports a titre of 1:40.

2. Absorption of complement with immune sera from rabbits

The test of Bordet-Gengou has not been very much used in the study of the dysentery bacilli, and the few contributions to the knowledge about it are contradictory. When the previous workers on this subject (Dopter, Haendel, Schroeter and Gutjahr, Amako and Kojima and others) disagree as to the value of the test, the cause may be sought either in inexactness of the reaction itself or in the technique of the worker. It was therefore thought to be of some interest to test this reaction as carefully as possible and with the same sera and strains used in the agglutination test.

As to the antigen, the earlier workers have mostly used an antiformin extract of the bacilli and claimed this to be better than the emulsion of bacilli. As regards the reaction in typhoid

it is proved by Brekke that the emulsion is quite as useful as the extract. But it is questionable, whether the destroying of the bacilli by antiformin and the following neutralization by acid is not apt to rob the bacilli of a good deal of their specificity, at any rate to a greater degree than the mere killing by heat. Accordingly an emulsion of bacilli in saline solution, heated for one hour to 60°C. has been used as antigen. The dose used has been one-twentieth of an agar slope culture (0.5 cc. of a suspension made in 10 cc. of saline solution).

As amboceptor have been used the same sera, used in the agglutination test. The sera have been titrated from 0.1 down to 0.00005 by halving each dose (0.1, 0.05, 0.025, etc.)

The dose of complement necessary for the hemolysis is in each case titrated out before the test itself. For the absorption test the dose is doubled and the solutions of dysentery serum and the emulsion of antigen, 0.5 cc. volume of each, are added.

The tubes are now placed in a waterbath at 37°C. for one and one-half hours, when 0.5 cc. of an emulsion of 5 per cent sheep blood corpuscles and the same volume of hemolytic rabbit-serum (1: 100) are added to each tube. After another one-half hour in the waterbath, the tubes are taken out and placed in the cold to sediment until the next day, when the titres of absorption are noted.

Using this method the absorption of complement has proved very valuable and specific. The results correspond very closely to those of the agglutination test. The strains isolated in this material were divided into the two main groups B and C, already mentioned. These groups were absolutely different in their reactions, while group B showed some, but not much relation to group A (the Shiga strain). Thus the absorption of complement separated the dysentery bacilli into the same three groups A, B and C that had already been identified with the agglutination test.

The results are put down in table 3.

TABLE 3

Absorption of complement in immune sera from rabbits (the figures signify the smallest dose of serum that gave positive reaction).

TESTED WITH			SERUM FROM NORMAL RABBIT	SERUM FROM RABBITS IMMUNIZED AGAINST			
Groups	Strains	A		B		C	
				Dys shiga	Danish group I O36		Danish group II F31
A		Dys. Shiga	÷	0 0002	0 025	0 0125	÷
B	Danish group I	O36	÷	0.1	0.0008	0.0016	÷
		F52	÷	÷	0.0032	0.0125	0.1
	Danish group II	F29	÷	0.0125	0.0032	0.0002	0.1
		F31	÷	0 0063	0.0032	0.0002	÷
		F57	÷	0.0125		0.0002	0.1
C	Group III	F40	÷	÷	÷	÷	0.0032
		F41	÷	÷	0.1	÷	0.0016

3. *The bactericidal effect of normal sera and of immunesera upon the dysentery bacilli*

The bactericidal method used in this investigation is that described by Neisser and Wechsberg, slightly modified by Haaland and Brekke. It is necessary to state the main lines of the reaction.

As immunesera have been used the same sera as in the agglutination and complement absorption tests. As complement, sera from normal guinea-pigs have been used. Before the reaction itself can be carried out, the complement must be titrated to find its own bactericidal effect upon the bacteria in question. This effect is called the total bactericidal effect. It is necessary to find this effect, as the dose used in the test itself must be lower than the lowest dose of complement that has any bactericidal effect itself without immuneserum. Simultaneously a test is carried out to find the activating power of the complement upon the immuneserum. The dose of complement employed in the bactericidal reaction must be smaller than the smallest dose that

gives a total bactericidal effect and larger than the smallest dose than can activate the immuneserum.

The antigen has always been derived from agar slope cultures of the bacteria, twenty-four hours old.

The reaction is carried out in ordinary test tubes, where the sera are titrated from 0.1 down to about 0.0000008 cc. by halving each dose. The stated necessary dose of complement is added to each tube, whereupon 1/8000 loop full (2 mm. diameter) of living bacteria + 2 drops of beef broth is added, and the tubes are put into the incubator, where they remain at 37°C. for three hours. Now 2 drops are taken out of each tube and inoculated into fluid agar, which is poured out into plate cultures and incubated for about twenty-four hours. The colonies are now counted. As titer is noted the smallest dose of serum, in which less than 100 colonies have developed.

We have to consider three different manifestations of the bactericidal effect of sera upon the bacteria, namely: (1) The effect of normal, active sera (the total bactericidal effect); (2) The effect of normal inactivated sera, added complement; (3) The effect of normal immunesera, inactivated and added complement.

1. A large number of fresh guinea-pig sera have been examined, some rabbit, and some human sera. With great regularity the sera have shown the same effect on the same groups of bacilli. The group A (the Shiga strain) and the group C (the Danish group III) have been very sensitive to the active serum, which always shows a high degree of bactericidal effect upon these microbes. Group B (the Danish group I and II), however, are less sensitive than A and C. Especially in group C this reaction seems to be of great value. It ought therefore to be used when a microbe, that evidently belongs to this group cannot be otherwise identified.

The result of several tests are put down in table 4.

2. Tested in normal, inactivated rabbit sera, plus the necessary dose of complement (titration of normal amboceptor) Group A and group B showed a fairly high bactericidal titre (down to 0.001 to 0.0002 cc. of serum), while group C was always

Total bactericidal effect of normal sera from guinea-pigs

GROUPS	STRAINS	NUM- BER OF SERA TRIED	BACTERICIDAL EFFECT ON THE STRAINS IN DOSES OF SERA				
			0 1	0.05	0 025	0 0125	0.0063
A	Dys. Shiga	4	+	+	+	+	+
		2	+	+	÷	÷	÷
B	Danish group I.....	O36	6	÷	÷	÷	÷
		F11	3	÷	÷	÷	÷
		1	+	+	÷	÷	÷
		F13	2	÷	÷	÷	÷
		1	+	+	÷	÷	÷
		F16	2	÷	÷	÷	÷
		1	+	÷	÷	÷	÷
		F51	1	+	÷	÷	÷
		1	÷	÷	÷	÷	÷
		F52	3	÷	÷	÷	÷
		1	+	÷	÷	÷	÷
		F55	1	÷	÷	÷	÷
	Danish group II.....	F60	1	÷	÷	÷	÷
		F61	1	÷	÷	÷	÷
		F90	1	+	+	+	÷
		F29	3	+	+	+	÷
		1	+	+	÷	÷	÷
		2	÷	÷	÷	÷	÷
		F30	1	+	+	+	÷
		F31	1	+	+	+	÷
		F	2	+	+	+	÷
		2	+	+	÷	÷	÷
		3	?	÷	÷	÷	÷
		F57	1	÷	÷	÷	÷
		F92	1	÷	÷	÷	÷
C	Group III.....	F40	3	+	+	+	÷
		1	+	+	+	÷	÷
		F41	1	+	+	+	+
		2	+	+	+	+	÷
		F71	1	+	+	+	÷
		F76	1	+	+	+	÷
		1	+	+	+	÷	÷
		F77	1	+	+	+	÷
		1	÷	÷	÷	÷	÷
		F79	1	+	+	+	÷
		1	+	+	+	÷	÷
		F86	1	+	+	+	+
		1	+	+	+	+	÷
		I	1	+	+	÷	÷
		V	1	+	+	+	÷
		VI	1	+	+	+	÷
		VII	1	÷	÷	÷	÷
		IX	1	+	+	+	÷

quite unaffected by the normal, inactivated serum.² Thus it must be assumed that normal rabbit sera contain amboceptor-like substances for group A and B, but no such substances for group C. Consequently a low titre of bactericidal effect of a rabbit serum against the former groups cannot be taken as any sign of specificity, while this must be the case in group C, even if the titre is low.

3. The bactericidal effect of the immunesera upon the various groups of bacilli showed exactly the same relationship between the groups as the agglutination and, the complement absorption tests. The strains isolated in our material fell into the same groups B and C as described before. In group B there was some difference between the strains corresponding to the Danish groups I and II, but this difference was not found important enough to split the group. Group C held the same isolated position as described under the agglutination and complement absorption tests. The bactericidal titres seem to be highest in groups A and B. But allowing for the normal bactericidal sensitiveness of these groups, one will find that the specific bactericidal reaction of group C is quite as high, though the doses of serum required to bring forth the bactericidal effect are larger in this group, where no normal sensitiveness supplements the specific one. As to group A (the Shiga strain), no specific reaction of this group in serum from group B could be found. On the other hand, group B showed a somewhat higher titre in the serum for group A than in normal rabbit sera, while group C was unaffected by this serum.

In sera from dysentery patients the same relationship between the groups was found as in rabbit sera. Especially in group C the reaction was of great value, and often more certain than the agglutination.

II. MORPHOLOGICAL AND CULTURAL CHARACTERS

The dysentery bacilli of the older types (Shiga, Y, Flexner and Strong types) always produce delicate, sharply contoured colonies on the plate cultures, of the same appearance as the col-

² Compare the absolute inagglutinability of this group in normal sera.

onies of the typhoid or paratyphoid bacilli. The new group (C Danish III), however, in this respect differs a good deal from the other groups. This group produces rather large colonies, a little denser than those of the other groups and showing a peculiar crenated irregular edge (fig. 1). The center is usually the densest part of the colony, the peripheral parts getting gradually thinner. Blue colonies of this type growing from a dysentery stool render it very likely that we have to deal with bacilli of this group. There may, however, be seen colonies of other types belong-

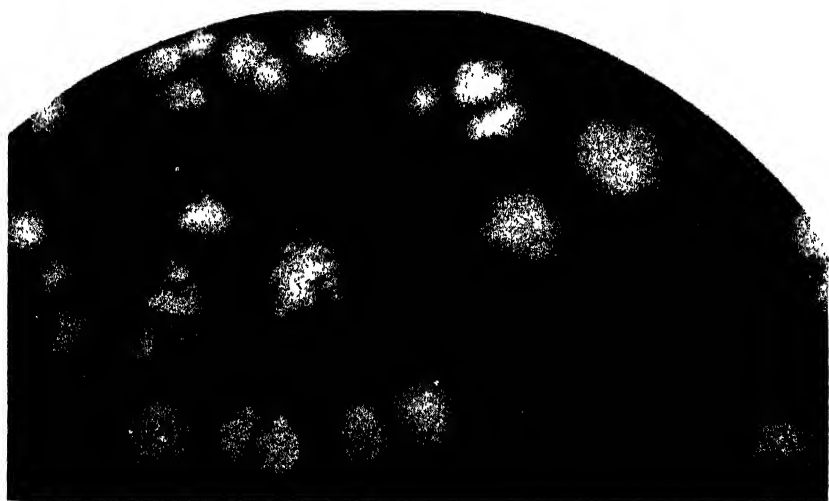


FIG. 1. COLONIES OF DYSENTERY BACILLUS OF GROUP III FORTY-EIGHT HOURS OLD, THIRCE MAGNIFIED

ing to this group. Sometimes we find them very small and dense, at other times large and dense with an edge growing out like a bunch of hair. Both these colonies show irregularities of agglutination, either being inagglutinable or showing spontaneous agglutination in saline solution. If cultivated for some generations on agar, these colonies will as a rule develop colonies of the ordinary type, both as regards agglutination and growth.

The growth of the isolated strains in mannit media always took place with production of acid without gas, thus characteriz-

ing the strains as belonging to the mannit fermenting group of the dysentery bacilli. In litmus milk there was as a rule a slight production of acid, but never coagulation.

The growth in lactose, maltose, sucrose, litmus whey and peptonized broth showed results, that make it necessary to deal with the different media separately.

1. Growth in lactose media

None of the strains showed any production of acid on the first plate culture from the stool after twenty-four hours of growth. A couple of strains belonging to group C, however, showed a peculiar behaviour on continued growth, that made it necessary to investigate more closely the fermentation of lactose by this group of dysentery bacilli. It was found that several of the strains of this group by prolonged growth were capable of producing acid in this sugar, as well in lactose agar (1 per cent) as in lactose broth. In one instance this was found in a first culture from a typical case of dysentery, that showed a clean growth of blue colonies of group C after twenty-four hours. After forty-eight hours of growth, however, the colonies had turned red, having produced acid as if it had been a culture of *Bact. coli*. In no strain belonging to our group B could any such faculty of fermenting the lactose be detected.

The use everywhere of lactose media for differentiating *Bact. coli* from the dysentery bacilli makes it imperative that one be aware of the possibility that bacilli of group C may to a certain extent be capable of fermenting this sugar or soon may learn to split it.

2. Growth in maltose media (see table 6)

It will be remembered that the fermentation of maltose-media had been the chief point of difference between the Y and the Flexner strains of the dysentery bacilli. This fermentation of maltose has been studied by the different authors partly in solid agar, partly in fluid media (beef broth or peptone water), and the results have been compared. Now the authors seem to pay no

no heed to the differences arising from the different media, although such differences certainly exist.

To compare the behavior of the same strains in solid and in fluid media 9 strains belonging to our groups B and C were inoculated into 1 per cent maltose-agar and maltose-broth, to which litmus was added, the media being slightly alkaline. Out of

TABLE 5

Bactericidal action of immune sera from rabbits (the figures signify the smallest doses of serum that showed bactericidal effect after addition of a convenient dose of complement

TESTED WITH		SERUM FROM NORMAL RABBIT	SERUM FROM RABBITS IMMUNIZED AGAINST			
Groups	Strains		A Dys Shiga	B Danish group I O36 Danish group II F31		C Group III F41
A	Dys. Shiga	0.0004	0 0000032	0 0002	0.0002	0.0002
B	Danish I strain O36	0.0008	0 0001	0 0000032	0.0000032	0.0008
	F52	0.0063	0 0001	0 0000063	0.0000125	0.0032
	F61			0 000025	0 00005	
	Danish group II. F29	0.0016	0.000025	0 00005	0.0000016	0 0016
	F31	0.0016	0.000025	0.000025	0.0000016	0.0008
C	Danish III strain	÷	÷	÷	÷	0.0001
	F40	÷				0.0002
	F41	÷	÷	÷	÷	0.0002
	F71	÷				0.0002

these 9 strains 3, belonging to group C produced acid as well in solid as in fluid medium, although the fluid cultures showed acid production more strongly and quickly than the solid ones. Of the 6 strains belonging to group B, 2 showed themselves as quite vivid producers of acid in the fluid culture, while the solid cultures remained alkaline. The others were non-fermenters both in solid and in fluid media.

This result shows that it is of little value to state whether maltose is fermented or not, when it is omitted to state, whether a solid or a fluid medium was used. It further shows that only when tested in fluid media can a non-maltose fermenting strain really be pronounced to be such.

The use of solid media is not advisable for another reason. The litmus sugar agar, has a great tendency towards being decolorized, probably on account of the absorption of oxygen by the microbe and the following reduction of the litmus. This decolorization begins at the bottom of the tube and ascends slowly to the top, leaving the agar uncoloured or dirty blue or red. In this condition it is often quite impossible to tell, whether the strain in question has produced any acid or not.

Not only the medium, but also the age of the strain has a great influence upon the fermentation of maltose. Already Lenz, Shiga, Krause and other investigators have stated this fact. In our material this influence of age was remarkable. In tests carried out about three years after the isolation of the first strains, several strains that were non-fermenters on isolation had acquired the faculty of vivid production of acid in maltose. Thus these strains had lost their first character of Y strains and had developed into Flexner strains according to the old nomenclature. In the younger strains, isolated from six months to one year previous to these tests, a growing tendency to ferment the maltose could be detected from one test to the next one.

Thus it is obvious that the dysentery bacilli, even if not fermenting maltose in the first culture, may acquire this faculty after some growth on artificial media. On the other hand two strains in this material which did ferment the maltose very vividly immediately after isolation, lost their fermenting power completely soon afterwards. Only a few strains remained non-fermenters all the time.

The increasing tendency to ferment maltose could be nicely demonstrated in the following experiment: Two strains of bacilli (one fermenting, one not) were cultivated in maltose-broth (1 per cent). From this medium the microbes were spread upon maltose-agar-plates.

In both strains the subcultures developed colonies that had fermented the maltose (turned the blue agar into red) and others that had not. These last colonies, that were perfectly typical in all other respects, were now put through new cultures of maltose broth and from these spread upon maltose-plates. Again the plates developed both kinds of colonies, but now the number of fermenting colonies increased, while the non-fermenting ones were reduced in number. After some new cultures the plates at last developed only fermenting colonies. Thus all the bacilli of these strains learned how to split the maltose after growth in a maltose medium.

When the fermenting of maltose is used in the grouping of the dysentery bacilli, the statement "fermenter" or "non fermenter" is made after a very short time of growth (twenty-four, forty-eight, and seventy-two hours). The fermentation, however, is not always finished at this time. Some strains are very slow fermenters, that require a longer space of time to produce acid enough to alter the reaction of the medium. Other strains certainly do produce acid in a short time, but in such a small quantity, that one feels inclined to put the strains among the nonfermenters. These strains consequently are neither strong fermenters, as the Flexner strains ought to be, nor are they non-fermenters like the Y strains, but take a position between these two types. If therefore, a just grouping according to the fermenting of maltose is to be achieved it is necessary to recognize a group of slight fermenters between the other two. But even this grouping can only be correct at the very moment of grouping. When examined sometime later on, namely, one may find that some of the non-fermenters have slid into the group of the slight fermenters, and some of the latter group into that of the strong fermenters. Consequently this mode of grouping is quite illogical.

Also if compared with the grouping based upon the serological characters the grouping according to the fermenting of maltose is illogical. From table 6 it will be seen that strong fermenters are to be found as well in our group B as in group C. Consequently these strains would have to be put into one group, if

TABLE 6

Fermentation of maltose and sucrose (the figures signify days of the experiments)

GROUPS	STRAINS	MALTOSÉ BROTH REACTION			SUCROSE BROTH REACTION	
		Alkaline	Slightly acid	Acid	Alkaline	Acid
B	Danish group I.....	O36		1-22	1-4	5-22
		F11	1-12	13-22	1-4	5-22
		F13	1-1		1-4	5-22
		F16	1-3	4-22	1-4	5-22
		F51	3-22	1-2	1-4	5-22
		F52		1-17	1-4	5-22
		F55		1-17	1-4	5-22
		F60	1-5	6-12	1-4	5-22
		F61	1-5 20-30	6-19	1-4	5-22
		F72		1-10	1-2	3-20
		F73		1-10	1-2	3-20
		F75		1	1-2	3-20
		F78		1	1	2-30
		F90		1-30	1-3	4-30
		F107		1-15		1-15
	Danish group II.....	F29	6-7	1-5	1-22	
		F30	6-7	1-5	1-22	
		F31	3-22	1-2	1-22	
		F57	3-22	1-2	1-22	
		F92		1-20	1-20	
		F99		1-20	1-20	
		F103		1-20	1	2-30
		F108	1-12		1-12	
		F109	1-12		1-12	
		F40		1-2	1-3	4-22
C	Group III.....	F41		1-2	1-3	4-22
		F71		1-3	1-2	3-20
		F76		1	1-2	3-20
		F77		1	1-5	6-20
		F79				
		F86		1	1-2	3-20
		F86			1-4	5-20
		F97		1-20	1-3	4-20

grouped according to the fermentation. It has, however, already been stated that group B differs so much from group C serologically, that it would be quite impossible to join these groups into one.

After this there can hardly be any doubt as to the worthlessness of the fermentation of maltose as a basis of grouping.

2. Growth in sucrose media (see table 6)

According to the classical scheme of the dysentery bacilli only the Strong type should be able to ferment sucrose. This dogma, however, was shaken by Sonne, who found fermentation of sucrose in both his groups I and III. Aronson found strains that fermented sucrose only and others that fermented sucrose and maltose to the same degree.

In our material several sucrose fermenting strains were isolated belonging to both our groups B and C. In the latter group all the strains were able to split this sugar and kept the faculty. Those of group B, however, seemed to have a tendency to lose this faculty, as they grew older. One lost it already after a couple of subcultures on agar, and another fermented sucrose after twenty-four hours of growth immediately after the isolation, but required four days in a test fourteen days later on. It must also be mentioned that an old Strong strain from Germany propagated in this institute at the time has no power of fermenting sucrose and consequently has lost the right to the Strong name.

In five experiments the fermentation of sucrose was studied and it was found, that the strains corresponding to group I of Sonne as a rule did ferment sucrose, those corresponding to Sonne's group II as a rule did not, while all belonging to our group C (Sonne's group III) were certain fermenters.

Thus the fermentation of sucrose is not so constant except in group C, that it can be fully depended upon. Consequently it has but little value in a scheme of grouping.

3. *Production of indol*

The production of indol has been examined in beef broth and peptone water, and the reaction carried out with the indol reagent of Ehrlich. The tests showed a distinct and constant difference between the groups. While nearly all the strains belonging to our group B did produce indol in a varying degree, not one of the strains of group C ever showed the slightest sign of indol.

As a rule the most vivid producers of acid were the poorest producers of indol and vice versa. This could be nicely demonstrated in experiments with maltose-broth, where strains, that did not ferment the sugar, had produced indol, while the contrary was seen in strains that did ferment the sugar.

4. *Growth in litmus whey*

The growth in litmus whey has in our material seemed to be of no use in the differentiation between the groups. As a rule the strains have produced acid after twenty-four hours of growth and stayed acid for the rest of the period of observation. On rare occasions there has been an alteration of the acid reaction after a week of growth into an alkaline one. Once only a strain from group C showed a third alteration of reaction. Having been acid the first week, the reaction was alkaline the second, and then again acid the third week. This is of interest, as Sonne finds the two first alterations regularly in all his strains of groups I and II (our group B), while all his strains of group III (our C) has shown the mentioned third alteration. It may be that this difference is to be accounted for by a difference of media, as Sonne has consistently worked with litmus whey from Kahlbaum, while the litmus whey used in our tests has been prepared in this laboratory.

CONCLUSIONS

The material of this investigation consists of 65 strains of dysentery bacilli, all belonging to the mannit fermenting types of the dysentery bacilli.

The old method of grouping according to the fermentation of different sugar media has been abandoned, as it has been found that this fermentation is subject to variations, that make the value of this grouping illusory.

Serologically our material falls into two groups, B and C.

The characters of B are as follows:

1. Production of rather small colonies quite like the colonies of *Bact. typhi*.

2. Production of acid in maltose and sucrose in fluid media. As a rule it is not found in the quite young strains, while the older ones very often show an increasing tendency of fermenting maltose.

3. Production of indol as a rule.

4. Serologically the group shows some relationship to the Shiga type, but none whatever to group C. The strains of group B show some individual variations in serological respects, but not important enough to justify the splitting of the group into more groups.

The characters of group C are the following:

1. Production of rather large colonies of a very typical aspect.

2. Production of acid in maltose and sucrose and occasionally in lactose.

3. No production of indol.

4. Serologically the group shows no relationship to the other groups of dysentery bacilli. The agglutination of this group is not observed in any normal sera or heterogeneous immune sera. The agglutination titres are rather low and the flocculi of the agglutinated emulsion are very fine. The strains of this group are considerably more sensitive to normal active sera from man and animals than the strains belonging to the other group. In immune sera from rabbits the group shows a very specific complement absorption test and bactericidal reaction.

Group B includes representatives of all the old types of the mannit fermenting dysentery bacilli. If then the Shiga type be counted, all the known dysentery bacilli should fall into three well characterized groups of bacilli. Above we have used the capitals A, B and C for these three groups. But as Kruse in Germany

already has employed these letters to signify different types of mannit fermenting dysentery bacilli, we propose to name the three groups mentioned above as group I, II, and III. Doing this, we adopt the grouping of Sonne with the alteration that Sonne's two first groups are united under the figure II, while the figure I is reserved for the bacillus of Shiga. Group III, however, will consistently be the same as group III of Sonne.

The new grouping I, II and III will be in full accordance with the chronology of the dysentery bacilli, the Shiga type being the

GROUPS	SEROLOGICALLY	CULTURALLY				
		Colonies	Mannit	Maltose	Saccharose	Indol
Group I (Bacillus of Shiga)	Specific. Very slight relationship to group II by agglutination, none by other sero-reactions	Rather small. Sharp, regular edge	÷	÷	÷	÷
Group II (Flexner, Y and Strong types)	Slight relationship to group I. Some individual variations of strains	Rather small. Sharp, regular edge	+ if observed the acid Once static	÷	÷ Or rarely +	+ (Seldom ÷)
Group III (Bacillus of Sonne)	Specific No relationship to other groups	Rather large. Uneven, irregular edge	+	+	+ (After some days)	÷

first one isolated, then the strains belonging to the old types Y, Flexner and Strong and at last the new type of Sonne. Further this mode of grouping will simplify the nomenclature by omitting all the names of authors and neglecting the smaller, insignificant variations of the strains that have given rise to all the different old "types." The new grouping will also bring the Shiga type closer up to the other dysentery bacilli, and thereby put an end to such illogical names as pseudodysenterybacilli, paradysenterybacilli, atoxic bacilli, etc. and render it unneces-

sary to use such long names as mannit fermenting or non mannitfermenting types. We propose hence only to talk about three groups of dysentery bacilli, namely:

Group I. The Shiga type (nonmannitfermenting, toxic type).

Group II. The Flexner, Strong and Y type (mannit fermenting, atoxic type, pseudodysentery bacilli, Sonne's groups I and II).

Group III. Group III of Sonne (the new member of the mannit fermenting types).

The main characters of these groups are as shown in the tabulated scheme on page 376.

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STUDIES OF THE DIPHTHERIA BACILLUS IN CULTURE¹

JOHN W. M. BUNKER

Digestive Ferments Company, Detroit, Michigan

Received for publication, February 16, 1919

The art of producing toxin from the diphtheria bacillus in culture is apparently very imperfect. While this procedure is one of the most important of the applications of bacteriology in the production of protective serum it has always remained an uncertain one. For every liter of toxin obtained of sufficient strength for immunizing purposes, many liters have been thrown out. Much effort has been expended endeavoring to overcome the uncertainty of the method by those interested commercially in the production of diphtheria antitoxin. In no other field of bacteriological endeavor may it more clearly be seen that the art of bacteriology is in its infancy. In spite of the best efforts to standardize procedure, failures are met with that cannot be explained. We are working with imperfect tools and largely in the dark. More scientific investigation of the essential nature of toxin and the mechanism of its production is necessary before we shall know where the mistakes occur which undoubtedly do occur. In the meantime, it is essential that every new advance in the science of bacteriology which may be applied to this problem, shall be investigated.

One of the most striking contributions to the science and art of bacteriology in recent years has been the demonstration by Clark and others of the effects of hydrogen-ion concentration upon the activities of bacteria in culture. By means of the hydrogen electrode or other suitable means for measuring ionized

¹ Presented before the Society of American Bacteriologists, in parts, December, 1916, December, 1917, and December, 1918.

hydrogen in solution we are able to determine in a much more exact manner than heretofore possible, the true acidity of culture media.

The art of culturing the diphtheria bacillus for toxin production has been built upon studies depending upon the use of litmus as an indicator of reaction. When one considers how imperfect is this manner of measuring reaction one wonders that favorable results were achieved at all. Successes have clearly been won in spite of crude methods.

THE HYDROGEN-ION CONCENTRATION OF THE MEDIUM

A study of the hydrogen-ion concentrations of culture media used for the production of toxin from the diphtheria bacillus was begun in this laboratory in 1916, and certain facts not hitherto mentioned in the literature were brought to light, which facts have, from time to time, been presented orally before the Society of American Bacteriologists. These observations have since been repeatedly confirmed, and it seems worth while to publish a record of them in order that others may have access to this information.

The first medium investigated was a simple meat infusion, enriched with Witte peptone because Witte peptone had always been used in past work. The infusion was prepared as follows:

Veal infusion base

Chopped veal.....	1 pound
Distilled water..	1000 cc.

Soaked overnight; next day heated to coagulate the proteins; juice expressed and made up to 1000 cc.

In our first experiment this veal infusion base was dispensed in Erlenmeyer flasks containing 200 cc. each. To three of these flasks Witte peptone was added to make 0.5, 2 and 4 per cent respectively of the total volume. One-half per cent of sodium chloride was added to each. The three flasks and the control flask (containing no peptone) were then titrated against phenolphthalein in the usual manner, using N/20 sodium hydroxide in boiling solution; and the hydrogen-ion concentration of each

flask was also determined by the hydrogen electrode. The results are given in figure 1.

It will be noted that the addition of Witte peptone to a veal infusion increases the titratable acidity of the medium in direct proportion to the amount of peptone added. At the same time ionization of hydrogen is depressed, resulting in a lowering of the hydrogen-ion concentration and a decrease of actual acidity. This phenomenon is explainable by attributing the lowering

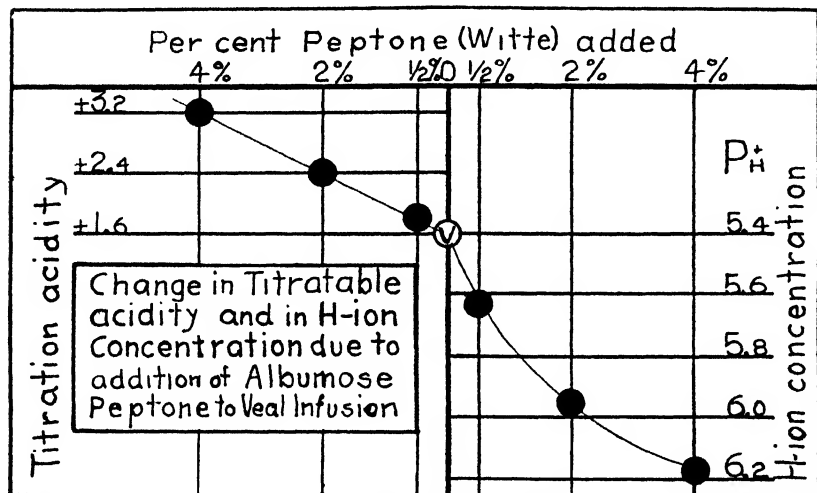


FIG. 1

of the actual hydrogen-ion concentration to the buffer action exerted by proteins in solution and especially by albumoses, in which Witte peptone is rich.

Here is a case, then, in which the method of determining reaction by titration is directly misleading, since the addition of peptone in varying amounts in this case really lowered the H-concentration although the titration value indicated that the acidity had been increased.

When the flasks as above prepared were "adjusted" with NaOH it was found that the changes in the titratable acidity and in hydrogen-ion concentration did not run parallel. Accordingly the following experiment was set up to indicate what

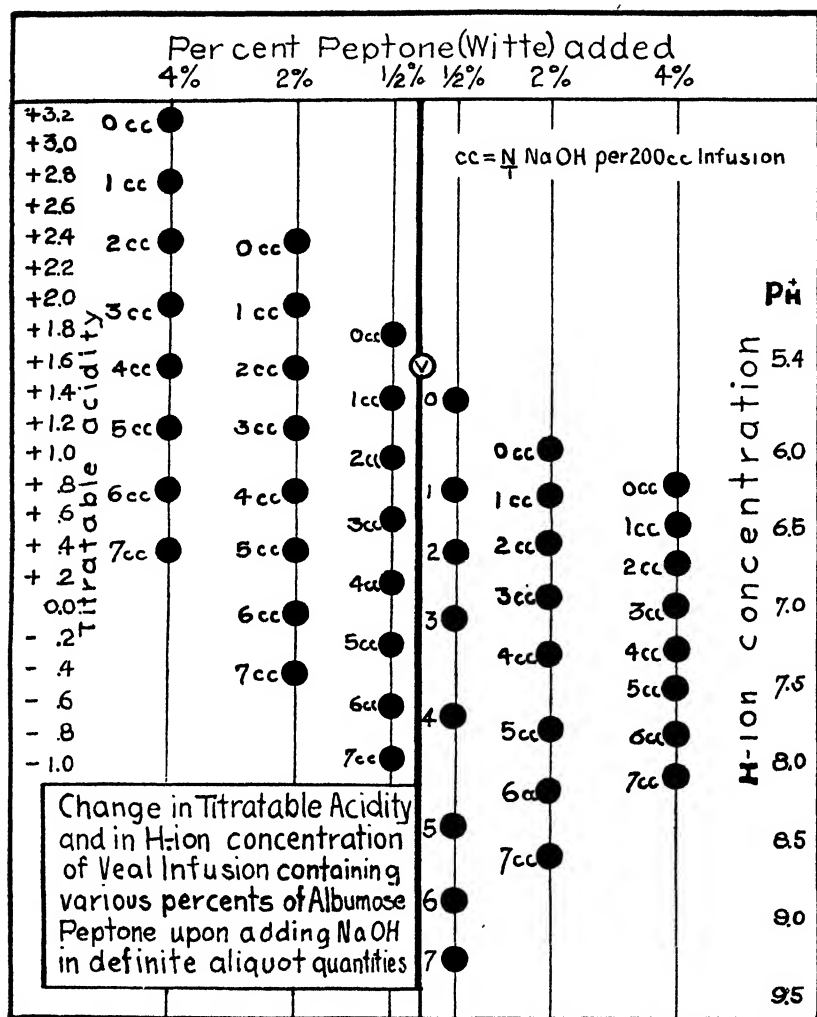


FIG. 2

changes take place in different media upon adjustment of reaction:

Three series of eight flasks each containing 180 cc. of veal infusion base were prepared. The series contained 0.5, 2, and 4 per cent of Witte peptone, respectively. To the members of each

of the series were added 0, 1, 2, 3, 4, 5, 6, and 7 cc. per flask respectively of normal NaOH and the volume made up to 200 cc. in each case with suitable peptone veal infusion. The flasks were then titrated and the H-ion concentrations taken. The results of these adjustments are plotted in figure 2.

It will be noted that the original veal infusion without peptone had a titratable value of + 1.6 and a pH value of 5.4. The acidity in all flasks was lowered by the addition of NaOH as measured both by titration and by the hydrogen electrode. The decrease in acidity as measured by titration was directly proportional to the amount of standard alkali added irrespective of the quantity of peptone in the medium.

The depression of H-ion concentration by the addition of alkali is regulated by the amount of peptone present; the more peptone present, the less the effect of a given aliquot of standard alkali. This is shown in figure 2, where the total change of H-ion concentration in 0.5 per cent peptone broth is indicated as from pH 5.63 to pH 9.20, whereas the addition of the same amount of alkali to 4 per cent peptone broth changed the pH from 6.16 to 8.06 only. Again the effect of buffer action is seen. In those media which contained more peptone, the addition of a given amount of alkali resulted in a smaller change in actual H-ion concentration than in those media containing less peptone and therefore less buffer.

This is another illustration of the fact that the true reaction of culture media is indicated by H-ion concentration measurements and that the values obtained by titration are merely apparent and are often misleading.

In order to show the effect upon true reaction due to sterilization of the media, the following experiment, T-47, is recorded:

Sugar-free veal infusion was used as a base. Witte peptone 2 per cent and 4 per cent was added, and in each case, 0.5 per cent sodium chloride. Two series of flasks were prepared, and the following laboratory record shows the changes which took place upon sterilization:

Table showing changes in reaction during preparation of culture media. Base-sugar-free veal infusion; 100 cc. per flask

FLASK NUMBER	WITTE PEPTONE	N/1 NaOH ADDED	REACTION BEFORE STERILIZING 15 MINUTES AT 15 POUNDS		REACTION AFTER STERILIZING 15 MINUTES AT 15 POUNDS	
			Hot titer	pH	Hot titer	pH
Stock	<i>per cent</i> 0	<i>cc.</i> 0	+2.7	4.96		
A-0	2	0	+3.4	5.69	3.1	5.69
1	2	1	+2.5	6.28	2.5	6.33
2	2	2	+1.3	6.98	1.7	6.89
3	2	3	+0.6	7.82	1.1	7.55
4	2	4	0.0	8.51	0.3	8.02
5	2	5	*	9.05	†	8.39
B-0	4	0	+4.0	5.97	4.5	5.86
1	4	1	3.5	6.43	3.4	6.43
2	4	2	2.3	7.08	2.8	6.83
3	4	3	1.6	7.56	2.0	7.39
4	4	4	0.5	8.12	1.2	7.75
5	4	5	†	8.63	0.3	8.19

* Distinctly alkaline.

† Faintly alkaline.

In the case of peptone veal infusion, the change in H-ion concentration upon sterilization is in the direction of increasing the acidity. In the more alkaline reactions the change is greatest. In the region of the optimum reaction for diphtheria growth, the change is equivalent to about 0.2 to 0.3 on the pH scale as borne out by experience. Always the change upon sterilization has been in the direction of increased acidity.

The results plotted in this table show that sterilization increases both the titratable and the actual acidity of media, also the fact that a given titratable value does not necessarily correspond to any given pH value, except that at the titratable value of "neutral to phenolphthalein" the pH value will, of course, be very close to the value $\text{pH} = 8.5$. This is necessarily true because unless the dissociation of hydrogen in the medium were of the value $\text{pH} = 8.5$, the medium would not be neutral to the particular indicator used—phenolphthalein. This

relation of titratable value to pH value has been pointed out before, but apparently is not generally recognized, namely that there is only one H-ion concentration at which the pH value and the titratable value of the solution for a given indicator will coincide, i.e.—the point at which the indicator undergoes the greatest dissociation and at which the color change takes place, a point which we commonly call the “neutral point” of that indicator.

TRUE REACTION AND GROWTH

The fact that reaction of culture media has an effect upon the growth and behavior of the diphtheria bacillus is too well known to merit discussion or to require support. It is true that reports of the effect of reaction of culture media have been based upon measurements of reaction made with a very uncertain point of reference, usually litmus. Nevertheless, empirical experiments have been made so many times that eventually we have been able to indicate how reaction as recorded by litmus does affect growth and metabolism under arbitrarily fixed conditions. Endeavors to duplicate any reaction studies made with litmus are subject to error because of the unavoidable differences in buffer content of various media. Nevertheless, the fact remains that it has been demonstrated that reaction does affect growth and metabolism, and therefore we know that reaction must affect toxin production.

In the light of present knowledge, it seems evident that this reaction effect must be due to hydrogen-ion concentration and therefore cannot be estimated by measurements of reaction which depend upon titration. To demonstrate this point beyond doubt, and to determine what are the limits of actual reaction or hydrogen-ion concentration within which favorable growth may be expected, flasks of veal infusion peptone broth, prepared and adjusted in an arbitrary manner as previously described, together with other flasks of similar medium, were together inoculated and incubated.

The flasks were observed daily, and observations of growth recorded. The most rapid development of pellicle took place

on those culture media having an initial reaction in the range pH = 5.6 to pH = 8.8. No attempt was made to sharply define these limits. Later on it was found that the diphtheria bacillus will grow beyond this range, both on the acid side and on the alkaline side after it has a start. The reactions mentioned were those observed to govern approximately the start of vigorous growth.

Previous to inoculation, both the H-ion concentration and the phenolphthalein titratable value of each flask were taken and recorded. Without repeating all of these results, some of the significant values are displayed in the following table:

Table showing the pH and titratable acidity of media which support growth of the diphtheria bacillus. All media are from the same veal infusion base

PEPTONE	HOT PHENOLPHTHALEIN TITRATION	pH	CHARACTER OF GROWTH
<i>per cent</i>			
0.5	+1.0	6.7	Fair
2.0	+1.2	6.9	Fair
4.0	+2.0	6.95	Fair
0.5	+0.2	7.65	Heavy
2.0	+0.4	7.7	Heavy
4.0	+0.8	7.8	Heavy
0.5	-0.2	8.4	Heavy
2.0	-0.1	8.4	Heavy
4.0	0.0	8.4	Heavy

It will be seen that the only place in which there is an agreement between the titratable value and the pH value is at the neutral point to phenolphthalein. At all reactions, the titratable acidities vary according as the buffer content of the medium varies.

It appears then that the reaction of a culture medium, as it affects growth of the diphtheria bacillus, cannot be accurately measured by titration, but that it is truly indicated by determination of the H-ion concentration of the medium.

THE EFFECT OF INITIAL HYDROGEN-ION CONCENTRATION
UPON GROWTH

Having demonstrated that H-ion concentration in the medium is one factor which controls growth, it was next in order to determine the optimum reaction in culture media as ordinarily prepared for toxin production.

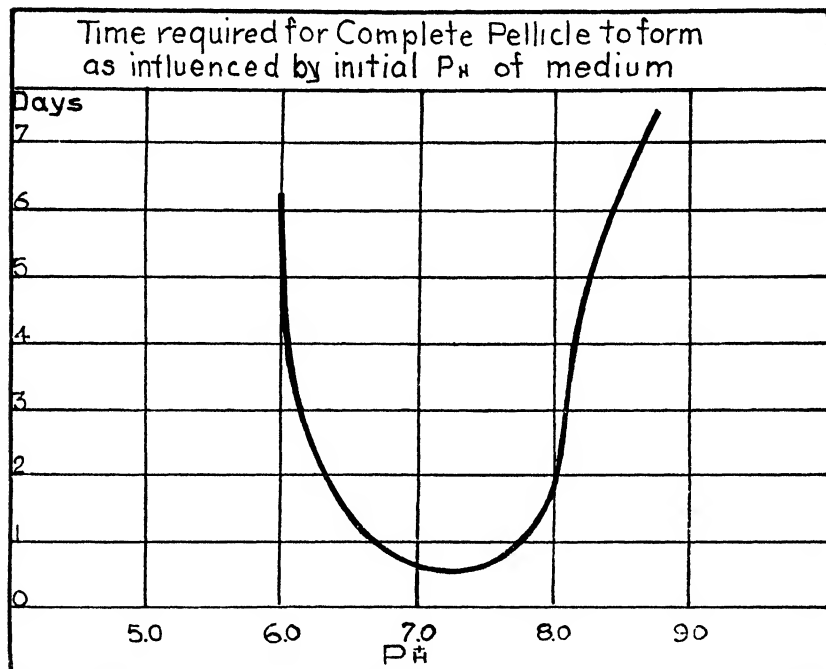


FIG. 3

The medium studied first was prepared as follows; Lean veal, chopped, was infused overnight in the ratio of 1 pound per liter of water, with a vigorous broth culture of *B. coli*, to remove the fermentable carbohydrates. After heating and clarifying, 2 per cent of Witte peptone and 0.5 per cent sodium chloride were added. No glucose was used. The medium was divided into 200 cc. portions in 10 ounce Erlenmeyer flasks in which it made a layer approximately $\frac{5}{8}$ inch deep.

After adjustment to various points over a range from pH 5.0 to pH 9.5 the flasks were sterilized, the final pH taken, and after inoculation, were incubated at 36°C. The time taken to form a complete pellicle over the whole surface was observed, and at the end of seven days, the relative thickness of the pellicle was estimated and recorded.

The results are plotted in figures 3 and 4.

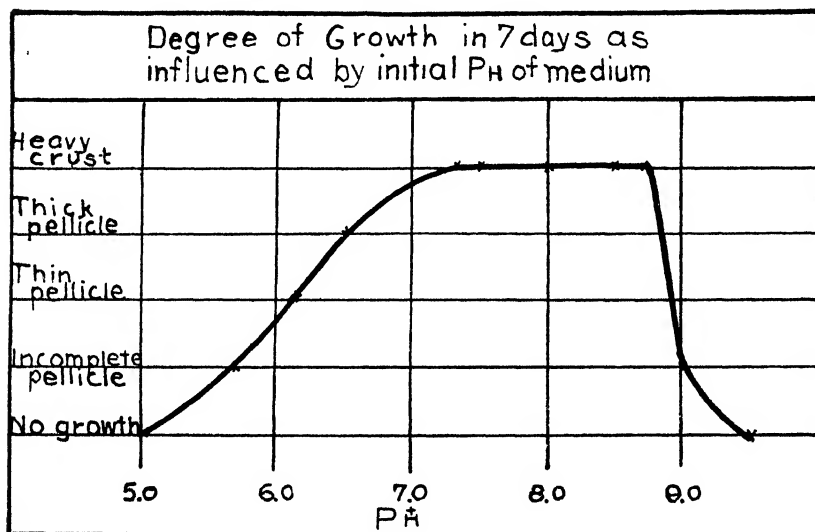


FIG. 4

It is apparent that the initial pH value of the medium affects the speed of growth as shown by the time required to form a continuous layer over the entire surface of the medium. Flasks in the acid end of the series with an initial pH more acid than 6.0 did not form a complete pellicle. At this reaction a thin pellicle formed slowly if at all. Between pH 6.7 and pH 7.7 a firm film would be formed in twenty-four hours, and between 7.0 and 7.5 it would be complete in from twelve to fourteen hours. At pH 8.0 the start of growth was delayed and the pellicle would not be completed until the second day. With the initial pH = 8.8 a week or more would elapse before the surface of the medium was covered.

The appearance of the growth too, differed with the initial H-ion concentration. Growths at the acid end of the series were in general thin and weak, while those at the alkaline end became thick and piled up. This is indicated in figure 4.

At the initial pH = 5.0 there was no growth. At 5.7 the surface growth at the end of a week was in patches and islands which did not cover the surface. At pH 6.0 a thin pellicle would sometimes be spread over the medium in seven days. At pH 6.5 a thin pellicle would be formed of firmer texture, and at 7.3 to 8.7 there would be formed a heavy white crust by the end of the week.

It would appear, therefore, that the optimum initial reaction for growth under the conditions described, lies between pH 7.0 and 7.5; and that the range 7.3 to 7.5 indicates the optimum initial reaction for the most rapid development of the heaviest pellicle.

THE CHANGE IN HYDROGEN-ION CONCENTRATION DURING GROWTH

It is known that the reaction of the culture medium changes during growth, usually becoming first more acid and then more alkaline. A study with the hydrogen electrode of daily reactions, shows considerable regularity and uniformity in this change which takes place, when expressed in terms of hydrogen-ion concentration. The nature of the change is dependent partly on the initial H-ion concentration and partly upon the substances in solution in the medium. This is necessarily to be expected since the reaction change results from growth, and since growth, and metabolism in turn, are profoundly affected by the reaction of the medium and by the nature of food substances available.

When the initial H-ion concentration is favorable for growth, there is first an acid rise in concentration followed by an alkaline reversion which proceeds rather directly to the region around pH = 8.0 and then more slowly to a limiting reaction of lower acidity. If the initial reaction is too acid there will be no growth, or at first a feeble growth accompanied by an acid rise which may proceed to an upper level which will be maintained

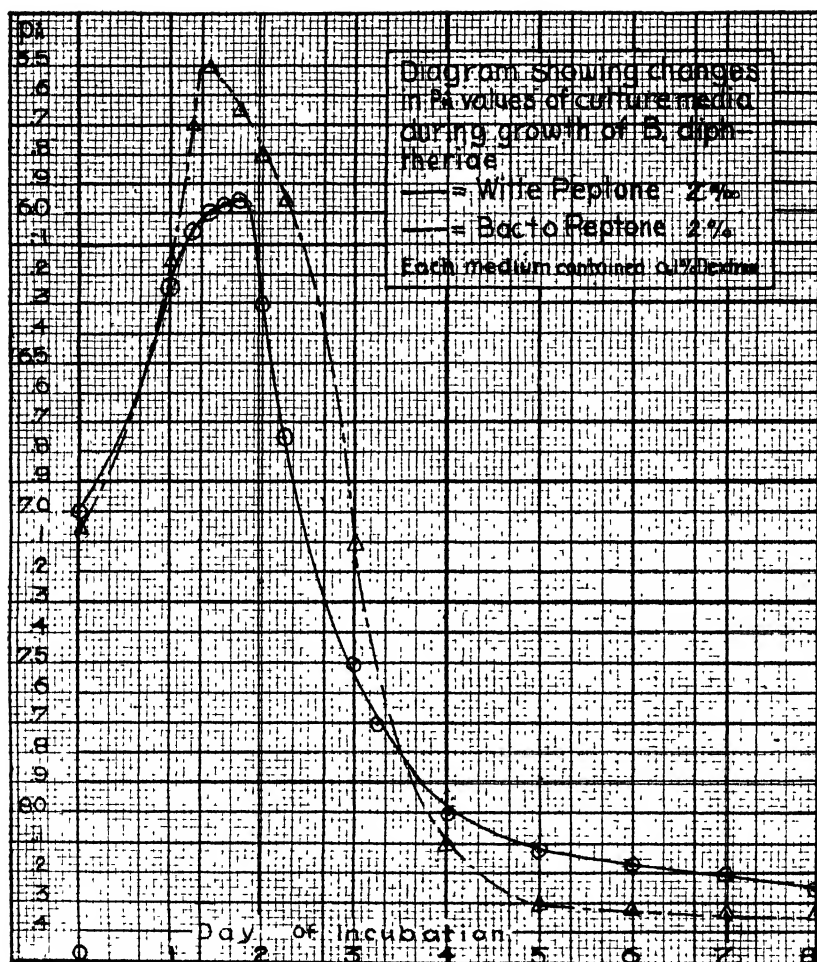


FIG. 5

until the culture dies. In such cases there is no alkaline reversion, and never in our experience have we obtained toxin from such conditions. Toxin has been gathered only when the typical reaction change from acid to alkali takes place.

Some typical daily reaction changes are plotted and reproduced in figures 5 and 6.

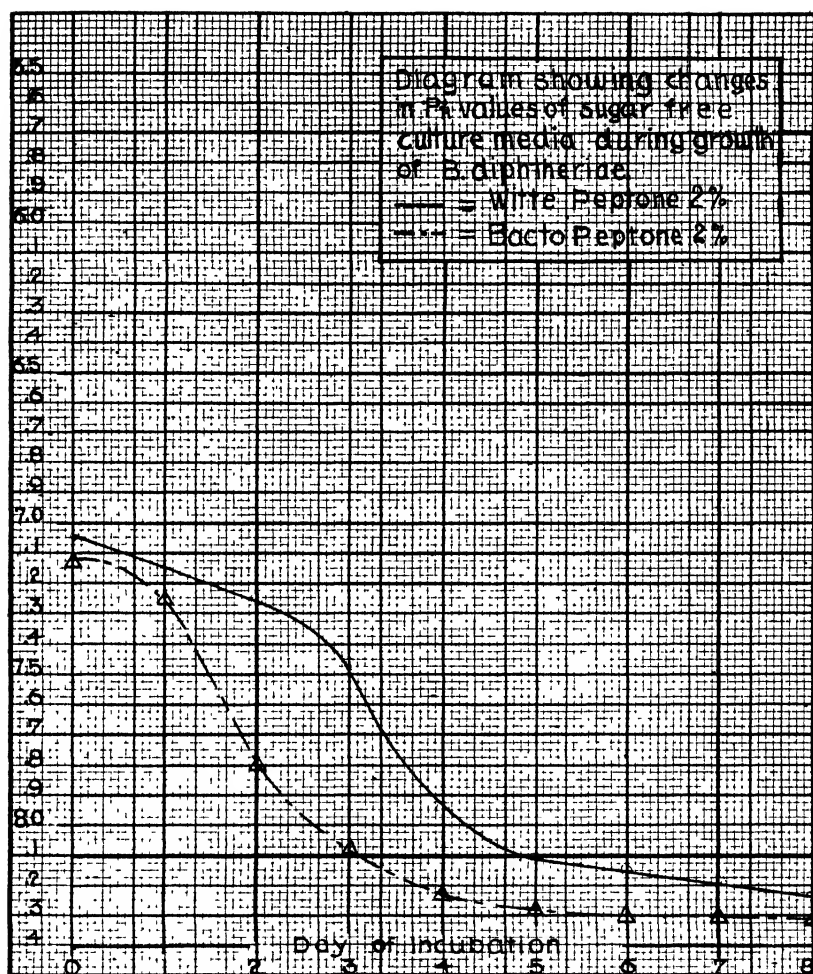


FIG. 6

In figure 5 is shown the plot of pH readings taken at various times over a period of eight days from two flasks, each containing the same veal infusion, each containing 0.1 per cent glucose, one enriched with Witte peptone 2 per cent and the other with Bacto-peptone 2 per cent. This diagram shows, in addition to the shape of the reaction curves formed under such conditions,

the effects of the nature of the peptone used. Witte peptone contains large quantities of albumoses. Bacto-peptone contains none. The acid rise in Witte peptone media is less than the acid rise in Bacto-peptone media under identical conditions. This is to be expected and is explained by the restraining action of the buffer substances in Witte peptone upon the ionization of acid. Although the acid ionization in the case of Bacto-peptone medium is greater than that with Witte peptone, the total change of reaction in Bacto-peptone goes to a more alkaline region than does Witte peptone. This again is attributed to buffer relations.

With different sized flasks and with other cultural conditions, curves of different shapes will be obtained, but in general a typical reaction curve in terms of H-ion concentration will follow the course marked out in diagram 5.

In figure 6 there are plotted daily reaction changes upon sugar free culture media, differing only from the previous flasks in regard to sugar content. The same lot of Witte peptone and the same lot of Bacto-peptone and the same quantities were used, the same incubation temperature was employed and the same culture.

The results obtained are typical of what one may expect under these conditions. So-called sugar-free media are often not free from fermentable substances, and if the media employed are not sugar-free there will always be an indication of this by a slight acid rise previous to the alkaline drop. In the two series which are reported, no acid rise was observed, but it should be remembered that no H-ion determinations were made during the first twenty-four hours of growth, so that a slight acid rise, if it occurred at this time, escaped our attention. The point which this diagram is intended to bring out, however, is that when a sugar-free medium is used and a carbohydrate-free peptone employed, there will not be the usual high production of acid previous to the alkaline reversion; rather the medium tends to become slowly alkaline and then abruptly alkaline and finally it will end up in the same general region of hydrogen-ion concentration reached by those media which contain sugar, this H-ion concentration being the lower limiting concentration for growth.

TOXIN PRODUCTION

With these facts at hand it seemed advisable that attention should next be turned toward an analytical study of reaction conditions during toxin production. The repeatedly confirmed observations of Park and Williams in 1896 concerning the apparent correlation between reaction changes in the substrate and toxin production offered a good starting point. Their work was repeated in part and again confirmed in the main. The record of H-ion reactions throughout these experiments gave a new set of criteria and a new method of specifying favorable conditions and unfavorable conditions insofar as reaction is concerned. For instance, the hydrogen electrode shows why these investigators found one optimum titratable reaction for media containing 1 per cent Witte peptone and another titratable reaction to be optimum when 4 per cent of peptone was used. The reason is that it is necessary in adjusting media containing different amounts of peptone, to add varying amounts of alkali to compensate for different degrees of buffer action in order to arrive at the one optimum H-ion concentration. Actually the reactions were the same or very similar, irrespective of the way in which they were adjusted, and in spite of the fact that titratable values were different.

A continued study of reaction changes during growth showed that there is a typical shape to the curve formed by plotting periodical readings when toxin is being produced, and that if any set of conditions causes the reaction changes to differ and form an atypical curve, toxin production is apparently affected adversely and may be entirely prevented.

In general, when toxin is produced the reaction changes follow the course indicated in figures 5 and 6, depending upon whether fermentable substances are present in the medium or not.

When for some reason the alkaline drop of hydrogen-ion concentration does not take place, toxin is not found. Toxin has been gathered, however, without any recorded preliminary acid rise. The writer is inclined to agree with the view that the addition of sugar merely speeds up initial growth and lends

momentum to the whole chemical reaction involved, and that if a sufficient amount of easily assimilable food substance is present in the medium to bring about this acceleration of growth, the addition of sugar to the medium is not beneficial.

In figure 7 are shown some atypical curves which were accompanied by failure to demonstrate any potent toxins at any time during the experiment.

That the nature of the "peptone" added has a marked influence on growth and the products of growth has been indicated. The effect of the buffer properties of peptone has already been pointed out. The effect of the kind of cleavage products supplied in the "peptone" content seems to have an even more important bearing on the development of by-products such as toxin. Either because of the form of the molecular structure or because of the nature of the food, certain groups of cleavage products stimulated toxin production. Whether this is to be regarded as explained by the agressin theory or whether these groupings stimulate certain metabolic processes indirectly resulting in toxin formation, or whether they supply the necessary atomic or molecular arrangements which enter into toxin formation, I do not know. At present we have only the observation of apparent correlation between the presence of certain groups of cleavage products and the formation of toxin, with the corollary absence of toxin when these groups are absent.

In determining this point, the line of procedure was first to try the available commercial peptones to see which were useful in developing toxin; next to investigate experimentally prepared peptones of different properties from different materials, and to see which of these would support toxin production.

A series of twenty different peptones, many of them made experimentally in this laboratory, were put on test at one time in small flasks such as were used in the preliminary work. H-ion concentration determinations were made daily and samples withdrawn each twenty-four hours. These samples were too many and too small to admit of filtration through Mandler filters so they were tri-cresolized with 0.5 per cent tri-cresol,

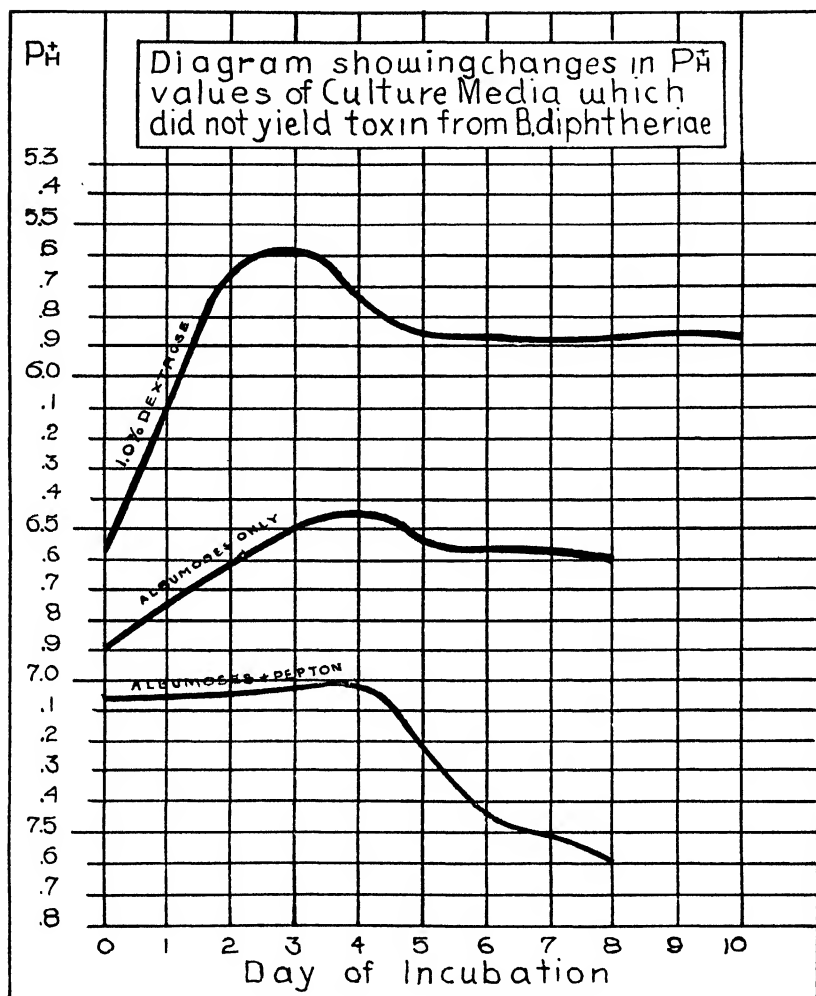


FIG. 7

shaken up, and allowed to stand in the ice-box for several days. Then the supernatant liquor was pipetted off and sealed into ampoules of about 30 cc. capacity. These were stored in the ice-box for a month or so, and were then shipped to one of the large biological manufacturing establishments to be tested on pigs for their minimum lethal dose strengths.

Of the series, Witte peptone developed toxin of the greatest potency, its minimum lethal doses being determined as a little better than $1/300$ th of a cubic centimeter. Other peptones developed a toxin of minimum lethal dose = $1/200$ th of a cubic centimeter or $1/100$ th of a cubic centimeter and many of the samples showed less strength than this. No results were regarded which were less than $1/100$ th of a cubic centimeter.

When the minimum lethal doses were plotted on the reaction curves, it was found that the most potent toxins occurred in one zone of H-ion concentration outside of which no strong toxins were harvested. Irrespective of the peptone medium from which toxin was obtained, this relation held true in this experiment.

In figure 8 are plotted the reaction curves of six culture media containing different peptones, from five of which toxin was obtained. The numbered circles indicate the points on the curves and the time of incubation at which samples were taken and tested for pH and minimum lethal dose.

In this diagram the peptones used were as follows: A = Witte peptone; B = Blood fibrin—tryptic digestion; C = uncured leather boiled with strong alkali; D = Bacto-peptone; E = Casein peptone—light digestion; F = primary albumoses from Witte peptone (A).

In the case of Witte peptone (curve A), samples taken on the first three days tested less than 0.01 cc. minimum lethal dose. From a theoretical standpoint it would be interesting to know the actual minimum lethal doses of each of these samples. This was a side issue in the investigation and was not carried to completion.

On the fourth day of growth, Witte peptone showed a toxic strength of 0.01 cc. which quantity killed a pig in three days. The pH of the medium at the time this sample was taken was 7.92.

On the fifth day of growth, Witte peptone showed a toxin of minimum lethal dose = 0.003 cc. and pH = 8.1.

On the sixth day the sample was of less strength, 0.005 cc. killing a pig in four days. The pH of the sample was 8.22.

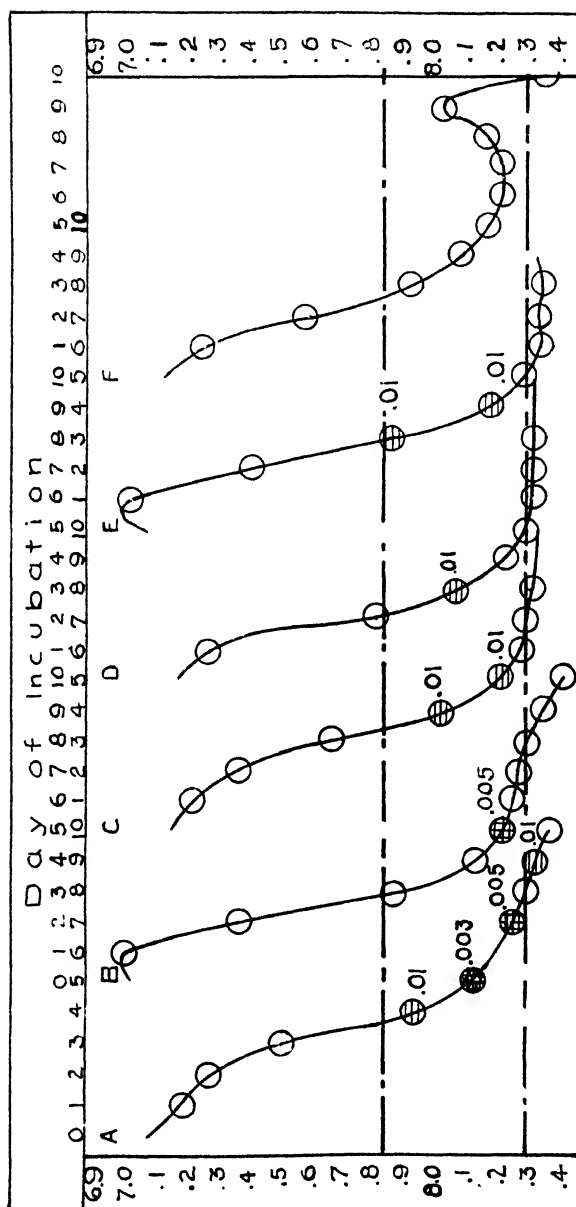


FIG. 8

The seventh day's sample was lost.

On the eight day the sample tested weaker its minimum lethal dose being only 0.01 cc. Its pH was 8.3.

These results with Witte peptone suggest that there is a point at which toxin development is at its maximum, before which and after which the potency will be lost. This point has been noted by previous investigators in terms of time elapsed in incubation. Present attempts to produce toxin call for incubation for a certain number of days before harvesting, still retaining the unit of time. This experiment suggests another possible criterion, namely, final H-ion concentration of the medium rather than age of the culture.

In curve B, again the strongest toxin was harvested on the fifth day of incubation at pH 8.2, the minimum lethal dose being 0.005 cc. Samples on the fourth day at pH 8.22 did not yield potent toxin.

In curve C, minimum lethal doses of 0.01 cc. were obtained on samples gathered on both the fourth and fifth days at pH 8.0 and 8.2 respectively.

In curve D, the sample gathered on the third day at pH 8.05 had a strength of 0.01 cc. Samples on the day previous and on the subsequent day had respectively pH's of 7.8 and 8.2 and neither one tested to a strength as high as 0.01 cc.

In curve E, two toxins of minimum lethal dose strength = 0.01 cc. were harvested on the third and fourth days respectively with H-ion concentrations of 7.85 and 8.1. Samples taken on the second, and on the fifth day from the same flask, did not show toxin of recordable strength.

In curve F, which is the curve obtained by recording the daily reaction changes in a medium enriched only with the albumose fraction of Witte peptone, obtained by half saturated ammonium sulphate precipitation, all samples taken failed to show any recordable toxic strength. Growth seemed rather sluggish and the reaction curve at the end of the run is atypical. It is not impossible that the peak on the ninth day of incubation may be the result of contamination. This was not looked for at the time and later it was not possible to determine the point.

Having plotted these results with the other fourteen of the series on a common scale, it became evident at a glance that all the strongest toxins had occurred at a given range of pH values on the respective curves, and that it would be possible to establish a zone which would include all the strong toxins and outside of which no potent toxin would occur. This zone was arbitrarily fixed by inspection as lying approximately between pH 7.85 and pH 8.25.

In the series of six experiments recorded and plotted in figure 10, it will be observed that no toxins of very great strength were obtained at any time, indicating that some fundamental condition in the experiment was at fault. However, insofar as we could determine, this condition applied to the whole experiment so that results may be tentatively at least taken as comparative. In this experiment, the strongest toxins obtained were in the case of curve A, on the fifth day, in the case of curve B on the fifth day; in the case of curve C on the fourth and fifth days with an indication that possibly a stronger point might have been found between the two; in the case of curve B on the third day with no indication that a stronger toxin could have been expected either before or after this time; in the case of curve E, equally strong toxins on the third and fourth days with nothing to indicate whether a stronger toxin might have been found between the two or after the fourth day sample; and in the case of curve F no strong toxin at all.

Regarding the first five samples which did yield some toxin, it will be seen that in this instance, even though the medium (with the exception of the peptone), the culture, and the incubation temperature are all identical, there is a difference in the time required in these five media to develop the strongest toxin. Regarding the initial H-ion concentration as affecting this, it will be noted that there is some difference in the initial concentration between the five samples examined, but sample C and sample D which both depart from the initial reaction of the other three to the greatest extent, are comparable with each other, and in the case of sample C, the maximum toxin production lies between the fourth and fifth days whereas in sample D,

it distinctly lies at the third day. This offers still further evidence that time of incubation is not the decisive governing factor in determining whether or not toxin will be found, but that reaction, which changes with a more or less constant relation to time in a given medium, is a better criterion.

The curves in figure 8 are governed by conditions present in small flasks where pellicle formation is completed very rapidly. In commercial production, larger flasks are generally employed with a resultant correspondingly increased period of incubation required to reach a given pH value. Under such conditions, the time required for incubation before the correct pH value is reached may be a week or more.

If different peptones are used, different time relations will accompany given pH changes, and if time alone is made the basis of judging when toxin is "ripe," it will be only by chance and in spite of technique that any peptone will give satisfactory results.

In other words, if it be true that the change in pH is affected by peptone, and is correlated to the change in strength of toxin during growth, and that such a correlation does not exist between time and toxin strength, then it is futile to try to duplicate results obtained from one product by applying the same technique in terms of time to another product.

Figure 8 demonstrates this point. If flasks made up with product D were grown for five days, which is the period of time indicated as suitable from experience with product A, whatever the best toxin production of product D might be, it would always be missed, since the maximum production with product D was at three days instead of five.

Another illustration of this is an experiment carried on under production conditions in which due to temperature irregularity a series of flasks which ordinarily should have reached the pH 7.85 in seven or eight days, did not do so until the fourteenth day, when with a pH of 7.83 the minimum lethal dose was 0.005 cc. . On the seventh day of this run at which time it would ordinarily be expected from past experience that toxin was ripe, the pH was only 6.8 and 0.01 cc. did not kill a pig. On the ninth

day with a pH = 7.41, 0.01 cc. did not kill. If this experiment had been run on a basis of time, the result would have been to throw away the broth which five days later was of sufficient potency to be useful. It is highly probable that this very thing has occurred many times in the past in the experience of commercial houses where batches of toxin broth have been discarded as having no strength, inasmuch as it has always been the practice of commercial producers as well as of laboratory experimenters, working with diphtheria toxin production to base the experiments upon the basis of time of growth, which, as it appears now, is not a constant factor. In the light of the preliminary work of this investigation which indicated the difference in buffer relations with different media and with different peptones, it will be seen that time and pH changes in the medium do not go hand in hand except for a given medium and for that rare case, a duplication of a given medium in every essential detail.

THE DEVELOPMENT OF A FAVORABLE MEDIUM

With the above observations in mind an attempt has been made to construct a medium which will support quick vigorous growth and at the same time permit a reaction change that will pass slowly through the critical zone, thus giving as much time as possible in which to gather the strongest toxin developed. Such a medium should be well buffered in the regions 7.8 to 8.2, as is 2.0 per cent Witte peptone broth.

The medium containing Witte peptone, as worked out by Park and Williams, will, with a favorable initial reaction, and the ordinary sized flasks, reach the critical zone and remain in it on the sixth, seventh and eighth day of incubation at 36°C. If the initial reaction is far from the favorable pH, as it may well be under existing methods of adjustment of media, time becomes an unreliable indicator of the proper point to gather the product.

It is apparent that many experiments have been discarded, not because toxin was not produced, but because it was not gathered when ripe before "deterioration", or because it was gathered before it had reached its greatest activity.

Broth may be prepared with true peptone and inorganic buffer which will follow in reaction change the favorable path, but we have never gathered strong toxin at any time from such media. Such media unbuffered pass rapidly through the critical harvesting zone, and if toxin is present at all it is there for a short time only, a matter of hours instead of days. It will be noted also that a medium enriched with Witte primary albumose alone, while it is highly buffered and stays in the critical zone for a long time, has not ever in our experience, yielded a potent toxin.

It would seem therefore, that not only must reaction changes be typical and not only must there be a suitable buffer substance in the medium, but there must also be some particular kind or grouping of food substance in order that toxin shall be developed.

An analytical study of Witte peptone and other peptone by an alcohol fractionation method, the technique of which will be published elsewhere, showed the presence of certain groupings of cleavage products in those peptones which gave good results, and the absence of these groupings in peptones giving poor results in toxin production.

Witte peptone has been separated arbitrarily into the following fractions: (1) Water insoluble; (2) soluble in water but insoluble in 50 per cent alcohol; (3) Soluble in 50 per cent alcohol but insoluble in 80 per cent alcohol; (4) soluble in 80 per cent alcohol.

These fractions have been tried separately and in combination to see if one or if any combination of them is of greater value in developing toxin than the whole group. The third fraction gives the most encouraging results, that portion which is soluble in 50 per cent alcohol and insoluble in 80 per cent alcohol. American peptones, some of them designedly so, are low in this fraction and rich in the 80 per cent soluble fraction, which is as it should be for purposes of growth stimulation.

An experimental product made from peptic digestion of meat and processed to eliminate the useless fraction of primary albumoses insoluble in 50 per cent alcohol, but retaining some of the group of polypeptides insoluble in 80 per cent alcohol, gives encouraging results.

A comparative fractionation of this product and Witte peptone made by the same arbitrary alcohol method referred to above, is given herewith:

	INSOLUBLE IN WATER	SOLUBLE IN WATER INSOLUBLE IN 50 PER CENT ALCOHOL	SOLUBLE IN 50 PER CENT ALCOHOL, INSOLUBLE IN 80 PER CENT ALCOHOL	SOLUBLE IN 80 PER CENT ALCOHOL
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Witte.....	7.29	26.16	50.14	15.86
Polypeptides.....	0.90	1.25	55.35	41.22

Growth takes place in a medium containing 2 per cent of this product which we shall call for convenience polypeptides, in a very satisfactory manner.

The reaction curve coincides with that of Witte peptone in 2.0 per cent medium over a period of seven days.

Toxin has been produced as often with this medium as with Witte medium, both in the laboratory over a period of twenty-three months, and under working conditions of commercial production in one of the large biological houses, and once with rather inadequate equipment in a government laboratory to meet an emergency during the war.

The most interesting test is that which extended over a period of one month, and was conducted with standard equipment and incubator space and facilities found at one of the large commercial biological laboratories. During this time, experiments were put on daily for approximately two weeks with the following results: Out of 12 starts with polypeptide medium there resulted minimum lethal doses as follows: One of 0.0025 cc., three of 0.005 cc., three of 0.01 cc., four less than 0.01 or doubtful. At the same time nine Witte controls were run which resulted in minimum lethal doses as follows: One 0.0025 cc., two 0.005 cc., two 0.01 cc., four less than 0.01 or doubtful. This is of course too small a number of experiments upon which to base any statistical analysis, but it will be noted that the percentage of failures to obtain toxin of usable strength in the case of the polypeptide medium is $33\frac{1}{3}$, whereas in a corresponding series

of Witte controls, the percentage of failure is 44. Such manipulation of small groups of figures, however, does not meet with our approval and we are content to draw the conclusion from this series and from other series that in the majority of cases the polypeptide medium is as useful in building potent diphtheria toxin as is Witte peptone.

The buffer content of the medium containing polypeptides is such that the reaction curve is reasonably flat during its passage through the critical zone of reaction. This is indicated by a record which follows:

Experiment 1918—O. Polypeptides medium, initial pH 7.5, temperature 36°C.

HARVESTED ON	MINIMUM LETHAL DOSE	REMARKS
Sixth day.....	0 005+	Pig died on sixth day
Seventh day.....	0 005—	Pig died in less than four days
Eighth day.....	0 005+	Pig died on sixth day
Ninth day.....	0 01—	
Tenth day.....	0 01—	
Eleventh day....	Lost	
Twelfth day.....	Less than 0.01	

Some toxin is obtainable under the conditions of this experiment on either the sixth, seventh or eighth day, but it is true that on the seventh day it is stronger than on the day previous or on the subsequent day.

The toxin so prepared has lasting qualities as shown by the following laboratory run:

Experiment 1918—Q. Toxin stored eighty days after gathering before testing

DAY GATHERED	WITTE	POLYPEPTIDES
Sixth day of incubation.....	0 005—	0.0020
Seventh day of incubation.....	0 0033—	0.0025
Eighth day of incubation.. . . .	Less than 0 005	0.0025

The technique used in obtaining these results is as follows: Lean veal is chopped and infused in tap water in the ratio of 1 pound per liter. The mixture of meat and water is made at the

latter end of the afternoon, and is warmed to about 40°C., in a large, open, jacketed kettle. To every 20 gallons of meat-mixture is added about 200 cc. of a broth culture of *B. coli*. In the morning the fermentable substances will have been largely removed if the mixture has not cooled down too much overnight.

Heat is then applied to coagulate the proteins and the infusion strained through several layers of cheesecloth and dispensed into 2-liter Erlenmeyer flasks which are plugged and sterilized in the autoclave for one-half hour at 20 pounds pressure. When it is desired to make up medium from this base, a flask is opened and if a precipitate has been formed in it through sterilization to such a degree as to be undesirable, it is first filtered through paper. Otherwise the desired quantity of veal infusion base is withdrawn and to it are added 0.5 per cent sodium chloride, 2.0 per cent polypeptides and sufficient sodium hydroxide to bring the reaction to $\text{pH} = 7.8$. This medium is then heated (in the autoclave for convenience) for about fifteen minutes at approximately 15 pounds. The purpose of this heating is to coagulate such material as will be precipitated by the change in reaction, and by heat. The precipitated medium is then passed through bibulous filter paper or thick cloth and dispensed in $\frac{1}{2}$ gallon rounded square amber glass bottles, 400 cc. per bottle. These are plugged with cotton, paper or cloth is tied over the necks, and they are sterilized in steam in the usual manner. When it is desired to inoculate the bottles, they are laid on their sides with the neck slightly raised, the covering is removed from the neck, the stopper withdrawn and the Bunsen burner played over the mouth of the flask for a second or two. It is then very simple to transfer a loopful of surface culture by means of a long-handled needle from the broth stock tube to the surface of the liquid in the bottle. We find that this method of floating on the inoculum is superior to pouring in a quantity of broth culture. Bottles are then stoppered and placed on their side in the incubator which is held at a temperature of 36°C.

By using this type of bottle instead of the round flask it is possible to stack bottles as high as incubator space will admit,

one on top of the other and still have plenty of air in each bottle over the surface of the medium to take care of the growth of the diphtheria organisms.

A film should spread over the surface of the liquid in twenty-four hours. Toxin at this temperature will be ripe in about seven days, but some toxin can be gathered on the sixth or the eighth day.

The procedure as outlined will give a medium which has a H-ion concentration of about $\text{pH} = 7.5$ after final sterilization.

CONCLUSIONS

1. The growth of the diphtheria bacillus in culture is influenced by the H-ion concentration of the medium. It is important, therefore, in adjusting culture media that the adjustment be made in terms of H-ion concentration.

2. It has been shown that the diphtheria bacillus will develop on peptone broth of an initial reaction between pH 5.7 and 8.7, and that when growth is started it may pass either of these limits.

3. It has been shown that for one strain at least, any initial reaction between 7.0 and 7.5 will give equally rapid development of a pellicle and that these two limits indicate approximately the range for optimum reaction as regards speed of development of pellicle.

4. It has been shown that at any initial reaction between pH 7.3 and pH 8.7 on ordinary media there will be formed in the course of about seven days, a pellicle of apparently equal weight after a more or less delayed start. It seems, therefore, that for the most rapid development of the heaviest pellicle, an initial reaction of pH 7.3 to 7.5 is most favorable.

5. The normal change of H-ion concentration during development of growth consists of a primary production of acid followed by a secondary alkali production which progresses until the pH 8.0 is reached, and then more slowly to the limiting concentration.

6. It has been observed that the harvesting of strong toxins has taken place within a definite H-ion concentration range

bounded approximately by pH 7.8 and pH 8.25. Witte peptone formerly used by bacteriologists is heavily buffered in this region. American peptones are not. This is suggested as one reason why the attempts to utilize American peptone with a technique based upon the past experience with Witte peptone have not been successful.

7. It has been shown that reaction conditions alone cannot govern toxin production. Besides favorable reaction conditions it is necessary also that suitable food substance be present in the media.

8. A peptic digest of meat having a content of polypeptides, insoluble in 80 per cent alcohol but soluble in 50 per cent similar to Witte peptone has given favorable results over a period of two years. In sugar-free veal infusion containing 2 per cent of this product and 0.5 per cent sodium chloride with a strong strain of P.W. No. 8, it is possible to obtain consistently usable toxins.

9. An initial H-ion concentration after final sterilization of pH=7.5 is indicated as favorable.

10. For every set of conditions, including the size and shape of containers the nature and the activity of the strain used, the incubation temperature, and other factors, it is necessary to work out by experience the time necessary to incubate cultures in order to obtain the most potent toxin. This may be done by means of following the daily H-ion concentration changes in a series of experiments under similar conditions. It will be found that the media, if prepared in the same way from the same materials, and the same conditions of culture applied, will reach a critical range of H-ion concentration between 7.8 and 8.25 approximately at a given time, and that once the time factor has been determined for the reaching of this critical zone, time may be used as a criterion for harvesting toxin from media made up in a similar manner thereafter.

THE GROWTH OF CERTAIN BACTERIA IN MEDIA OF DIFFERENT HYDROGEN ION CONCENTRATIONS¹

BARNETT COHEN AND WM. MANSFIELD CLARK

*From the Research Laboratories of the Dairy Division, Bureau of Animal Industry,
United States Department of Agriculture*

Received for publication, March 26, 1919

The literature of bacteriology is replete with statements that the "reaction" of a culture medium is important, but important for what particular phases of growth or for what specific processes of metabolism is not yet known in much detail. Recent investigations have made clear certain of the more general aspects of the subject and have demonstrated the value of hydrogen ion determinations; but circumspection must be exercised, and much additional experimental data will be required before the mechanism of the phenomena now known can be explained. The influence of the hydrogen ion on cellular multiplication is probably far from being one of those simple matters which we like to call fundamental; yet it is obvious that we must seek detailed information concerning this phase before studying intensively the metabolism of a culture as a whole over long or short periods and at various pH reactions.

Much of the work contributory to the subject has been reviewed in previous papers from this laboratory (J. Bacteriol., 2), and in subsequent papers by others. In many of these contributions the authors have dealt chiefly with the lethal or limiting pH, but in others such as those of Shohl and Janney (1917) and of Dernby and Avery (1918) we find data upon rates of growth within a range of pH favorable to growth. The reports of Bunker (1916-1917) on studies with the diphtheria

¹ Published by permission of the Secretary of Agriculture.

A report of this work was read at the Washington meeting of the Society of American Bacteriologists, December, 1917.

bacillus, of Cole and Lloyd (1917) on the gonococcus, of Cullen and Chesney (1918) on pneumococci, of Fred and Loomis (1917) on soil bacteria and of Gillespie (1918) on an organism (causing "potato-scab") whose response to pH is of considerable importance to potato growers, as well as several other recent investigations have all served to outline the field for a survey, the extension of which is greatly needed.

In the present investigation we have attempted to narrow the problem with the hope of obtaining more quantitative results. We set out to determine the influence of pH upon rates of growth during that period in the life history of a bacterial culture commonly known as the period of logarithmic increase. The investigation assumed the aspect of a preliminary survey until it became evident that some uncontrolled factor was shortening the period of logarithmic growth to a disconcerting degree. No attempt was made to remedy this by empirical readjustment of conditions. We continued with the materials and the methods with which we had started because the results seemed to be suggestive and because it seemed desirable to have at least a preliminary survey before attempting a special and systematic search for the unknown factor or factors involved.

The organisms studied were: *B. coli*, laboratory culture 22 fg; *B. aerogenes*, laboratory culture 22aax; *B. dysenteriae* (Flexner), American Museum No. 110; *B. dysenteriae* (Shiga) originally from Kruse, furnished by the Rockefeller Institute; *B. proteus*, furnished by Miss Bengston, United States Hygienic Laboratory; *B. alcaligenes*, American Museum and *B. bulgaricus*, laboratory culture 39a.

The organisms were cultivated in a peptone phosphate broth for multiplication studies and upon nutrient agar for counts. The broth consisted of 1 per cent "Difco" peptone and 0.5 per cent K_2HPO_4 to which was added HCl or NaOH to obtain the desired pH. The pH values were determined colorimetrically with the indicators and the procedure described by Clark and Lubs (1917a) and with occasional electrometric control (Clark, 1915a). The nutrient agar was adjusted to pH 7.1. The inocula consisted of aliquot volumes of a culture incubated

eighteen hours at 37°C. The medium for this culture was of the same composition as that inoculated, and was adjusted initially to pH 7.1. The multiplication studies were made in 200 cc. portions of the medium warmed to 37°C. before inoculation and held at this temperature during the experiment. Plates were held seventy-two hours at 37°C. before being counted. Frequent tests of pH during an experiment showed that the change in reaction was insignificant during the short period considered in the calculation of rates of logarithmic increase. When it is necessary to state the results of counts taken over periods as long as five to ten hours we shall use the pH value found in each instance at the time a count was made.

By plotting against time the logarithms of the counts it is possible to obtain by inspection of the curve an estimate of the period within which the increase in viable cells approaches the logarithmic law. The counts found during the period so estimated were introduced into the equation: $b = a \times 2^n$. Here b represents the number of organisms at the end of a period T , a the number at the beginning of the period, and n the number of generations during this period. When the length of the period T is expressed in hours, $\frac{n}{T}$ gives the number of generations per hour. These equations are approximate but quite adequate when we consider the large experimental error involved in counting the plates.

It frequently happened, especially at the more extreme pH values, that no three counts fell on a curve which indicated true logarithmic increase. In such cases we have considered the highest average rate computed from three or more successive counts to indicate the magnitude of any logarithmic increase which may have occurred. As an example of the series of values found there are shown in figure 1 the generations per hour plotted as ordinates and computed from counts of *B. coli* cultures held at the pH indicated by the abscissae. The negative rate shown at pH 4.6 means that a logarithmic decline in numbers was observed for a short period in this case.

Not all the experiments furnished data giving such a smooth curve as that shown in figure 1, but in almost every case the same type of curve was obtained with the other organisms. In each there was very plainly evident a broad zone within which relatively high and more or less uniform rates of reproduction were found for short periods. It is also very clear that at the borders of such a zone a comparatively slight shift in pH produces a profound effect.

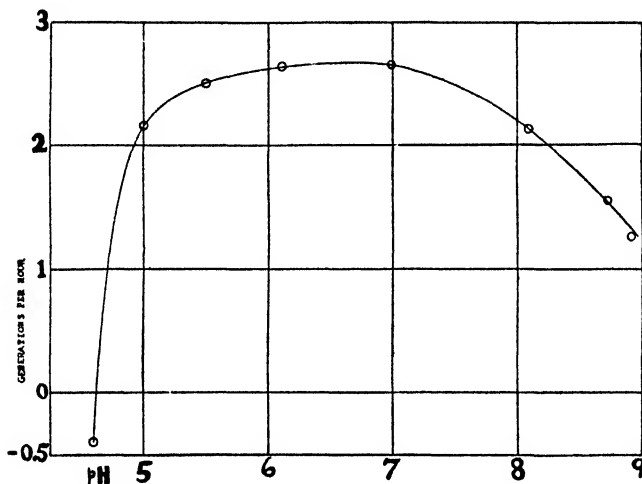


FIG. 1. RATES OF GROWTH OF *B. COLI*

Average generations per hour plotted as ordinates against pH as abscissae

There is some evidence, too incomplete to dwell upon, that the slope of the curve for *B. alcaligenes* is not abrupt on the acid side as it is for the other organisms studied, and that we have in this case a type of curve which is in this respect distinct. Since there is so plainly evident a broad zone of pH within which relatively high maximal rates of growth were observed for short periods, we may conveniently summarize all the data on this point in table 1.

The chief effect of reaction in so far as we have to do only with the period of maximal rate of increase seems to be confined to the limiting extremes. This is indicated in figure 1, and again in

TABLE 1

Generations per hour for period of logarithmic increase within pH zones indicated

ORGANISM*	pH RANGE	AVERAGE GENERATIONS PER HOUR
<i>B. coli</i>	5 0-8 1	2 5
<i>B. aerogenes</i> ..	4 7-8 4	2 7
<i>B. dysenteriae</i> (Flexner)....	5 6-8 3	1 6
<i>B. dysenteriae</i> (Shiga) .	5 5-8 5	1 1
<i>B. proteus</i> .	6 5-8 8	1 5
<i>B. alcaligenes</i> ..	6 9-9 7	0 9

* *B. bulgaricus* is not included in this table because it was studied in a medium of different composition, and the results may not be comparable with those of the other organisms.

table 2. In table 2 are shown first the pH at which the numbers of viable organisms declined,—the decline being expressed as generations per hour with a negative sign. Next are shown the maximal rates of increase at the highest acidity where definite increases were observed. Similarly, the conduct of the organisms in alkaline media just within, and in two cases without the zone where growth was observed are shown in the last columns.

TABLE 2

Rates of increase at pH values indicated

	ON ACID SIDE				ON ALKALINE SIDE			
	pH	Rate*	pH	Rate	pH	Rate	pH	Rate
<i>B. coli</i>	4 6	-0 4	5 0	2 2	8 9	1 3		
<i>B. aerogenes</i> .	4 4	-1 0	4 7	2 6	9 0	0 3		
<i>B. dysenteriae</i> (Flexner)...			4 8	0 8	8 7	0 9	9 1	-5 6
<i>B. dysenteriae</i> (Shiga) ..	4 7	-0 6	5 3	0 9	8 8	0 3		
<i>B. proteus</i> ..	5.4	-1.7	5 6	0 9	8 9	0 2	9 0	-0 1
<i>B. alcaligenes</i> .	5 5	-0 3	5 7	0 2	9 7	0 8		

* In generations per hour.

There are some points of special interest to be noted in the fact that, while the organisms can multiply rapidly for short periods in media having a considerable range of pH, a slight change at the border of this zone determines a definite decline in numbers. This illustrates a theoretical principle discussed briefly by Clark and Lubs (1917a) who pointed out that substances whose dis-

sociation is influenced by the pH of a solution would be affected but little within wide zones of pH, and profoundly affected by slight changes at a border or borders of such a zone. In the case now under consideration, the change in the conduct of the cells is more abrupt than might be expected and is, therefore, of special theoretical interest.

Since the change is so sharp it might be supposed that we have in the study of reproduction a method capable of showing a definite limiting pH—a method which is applicable where others are not. For instance, it is specially interesting to note that in our present series of experiments the critical pH for *B. aerogenes* occurs at about 4.5. This organism decomposes organic acids so readily (Ayers and Rupp, 1918) that it is difficult except under accidental or skillfully controlled conditions to force the culture by sugar fermentation to so high an acidity as pH 4.5. It was this fact that Clark and Lubs (1917) took advantage of in designing the so-called methyl-red test. We have obtained values as acid as pH 4.9 by the fermentation method under suitably controlled conditions, but for the reason just stated have never felt as certain of their significance as now when seeking the limits for *B. aerogenes* by the present method. The reproduction studies indicate that *B. aerogenes* and *B. coli* are influenced about alike in their reproduction by the same pH.

On the other hand, the value so obtained can not be considered as a well-defined constant. The fermentative limit was discussed by Clark and Lubs (1917b) who prefer to consider the values experimentally found in particular cases as indicating a limiting zone. That such a limiting zone in the case of reproduction may become wide is shown by some experiments in the present series where adjustments of pH were made with acetic acid in place of hydrochloric acid. Distinct differences were observed. For instance at pH 5.0 when obtained with HCl, *B. coli* grew well for a time; but at pH 5.45, when obtained with acetic acid, the same organism barely held its own and finally declined in numbers. The same difference in conduct was observed with other organisms. We did not at that time pursue this subject further in order to determine the conduct of *B. coli* in acetic acid

media of slightly higher pH, but some months later Dr. Sherman kindly repeated the experiment with this end in view. He found in media adjusted with acetic acid that at pH 5.5 there was a slight decline in numbers extending over the first sixteen hours of observation—thus confirming our earlier work. No cloud indicating growth ever developed in this medium. At pH 5.7 he found an almost stationary value for the number of viable cells counted during the first sixteen hours, but at the end of twenty-four hours he found a large increase. At pH 6.0 the same thing occurred except that the increase began at the tenth hour; and at pH 6.2 the increase began between the second and fourth hours. In this case, then, the narrow pH zone 5.5–5.7 may be considered critical, while in the former case where the medium was adjusted with HCl it was 4.6–5.0.

We come now to a brief consideration of the “unknown” factor to which we have already alluded. The question naturally arises: Is it truly an unknown factor, or merely one which we should have recognized and controlled? We are not unmindful of the considerable body of literature bearing upon inhibition of bacterial growth, but we know of no points which are in our opinion sufficiently well established to permit of application to this admittedly brief and preliminary survey. Mature deliberation has led us to the conclusion that it is preferable to consider that we have yet to discover the main cause of the sudden inhibition of growth. Rather than repeat our experiments by a hit or miss method of improvement it seems wiser to let the present results stand for what they are worth until such a time as we can recast the procedure intelligently.

In common with other observers we found the period of logarithmic increase to be of short duration. Evidence of inhibition often appears within four hours after inoculation. Can this be due to an accumulation of by-products of metabolism? To show how difficult the solution of this problem may become by an unsuitable choice of conditions, we shall first describe an experiment with a *B. coli* culture grown in 1 per cent Witte peptone, 0.5 per cent K_2HPO_4 and 1 per cent glucose, and in which pH determinations and counts of viable bacteria were

made simultaneously. The results are plotted in figure 2, where the abscissae are hours, and the ordinates logarithms of the numbers of bacteria (scale at left), and pH values (scale at right). The curve for the sugar-free medium is included for comparison and will be considered presently.

Between the fifth and tenth hours the increase in the number of viable bacteria followed the logarithmic law, but between the tenth and the fourteenth hours inhibition of growth became evident. In this period the pH of the medium approached that

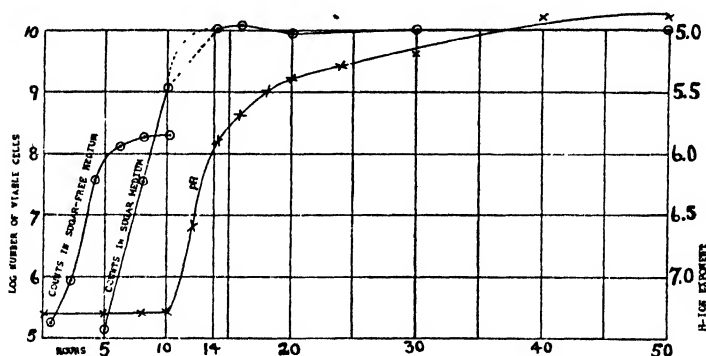


FIG. 2. COMPARISON OF THE GROWTH CURVE OF *B. COLI* IN PLAIN AND GLUCOSE MEDIA; AND THE ACID DEVELOPMENT IN THE GLUCOSE MEDIUM EXPRESSED AS pH

The abscissae give the time in hours. The ordinates: at left, logarithm of number of viable cells; at right, pH (hydrogen ion "exponent")

region where we have found acetic acid to inhibit growth. Was the check in growth in this instance due to the pH or perhaps to acetic acid which is a product of the sugar fermentation of *B. coli*? The question might have received a clearer answer had frequent and accurate counts been made between the tenth and the fourteenth hours; but the margin of safety in drawing conclusions would have been narrow.

For purposes of comparison there are plotted in figure 2 the logarithms of the numbers of viable *B. coli* in a sugar-free medium whose pH remained practically constant. It is evident from inspection of this curve that a severe check in growth took place early and that it was due to some factor other than pH.

We have then this peculiar situation. As is indicated by the flattening of the pH curve as well as by other facts, the *fermentative activity* in the glucose medium is checked when *B. coli* brings the culture to a zone of pH close to that at which HCl checks growth; but the *growth* of *B. coli* is checked when as it happens in this case, the pH approaches that at which acetic acid, a product of its sugar fermentation, can be made to check growth.

There is considerable evidence, some of which has been presented in previous papers from this laboratory, which points to the conclusion that under ordinary cultural conditions it is the pH which exerts the greatest influence upon acid and gas production by *B. coli*, and that other inhibitory conditions are of secondary importance. It is possible that these other inhibitory conditions may have a strong effect upon the processes of reproduction, but may not make themselves felt to any great extent upon the fermentative activities. When we turn to the experiment with the sugar-free medium in which the pH was held practically constant and favorable to growth, we find growth seriously affected after the fourth hour. Yet the number of viable cells at this moment was but 5 per cent of that in the sugar medium when the growth curve of the latter fell from the logarithmic rate. It is to be strongly emphasized that a comparison of the *effect* of the products in the two cases is unfair, but the alternative assumption that the much smaller number of organisms in the one case could have produced a more active *quantity* of by-products in a shorter time is to be doubted.

It may be suggestive to note that, while the rates of logarithmic increase taken early in the course of the growth curve furnish no distinct optimal pH, those taken over longer periods do. Cole and Lloyd found that in simple media gonococcus required a narrow zone of pH, while in "hormone" media the pH zone within which growth was good was comparatively broad. It is possible that in the simple medium there were rapidly produced those conditions which appear only later in the more complex media.

Chesney (1916) and many others have already dealt with the inhibition of growth, but we feel that much remains to be clarified. We shall not discuss it further, having mentioned it merely

to show that we recognize a difficulty which seriously hampers the clear analysis of our data. However, it seems clear that the influence of this unknown factor will tend to obscure the influence of the hydrogen ion concentration, and that the differences observed indicate at least submaximal effects of pH.

The only variation between the media in the main series was, so far as we know, in the quantity of HCl or NaOH added, but there are two other factors which may have some bearing on the results, and which were not investigated. In the first place, although our inocula contained numbers of viable cells of the same order of magnitude, these numbers were large. Slator (1913) has shown that in the cultivation of yeast cells it is better to use small numbers if logarithmic increases are to be observed. In the second place, attention should be called to the fact that the organisms employed do not grow well on sugar-free media if the air is removed by evacuation. Our experiments were conducted in 200 cc. portions of the medium, and although exposed to the air and shaken frequently before the removal of the samples, there may have been a gradual and irregular transition from aerobic to partially anaerobic conditions.

There still remains for consideration the influence of reaction upon periods other than that of logarithmic increase, as well as upon the whole course of the growth curve.

We noted with *B. coli*, *B. aerogenes*, *B. dysenteriae* (Shiga), *B. proteus* and *B. bulgaricus* that the time of maximal rate of growth was delayed until a late hour in the more alkaline media. With *B. coli* the periods of maximal rate of increase occurred as follows:

pH _a	PERIOD
	hours
5.0	3-5
5.5	3-5
6.1	2-4
7.0	2-4
8.1	2-4
8.7	8-10
8.9	10-12

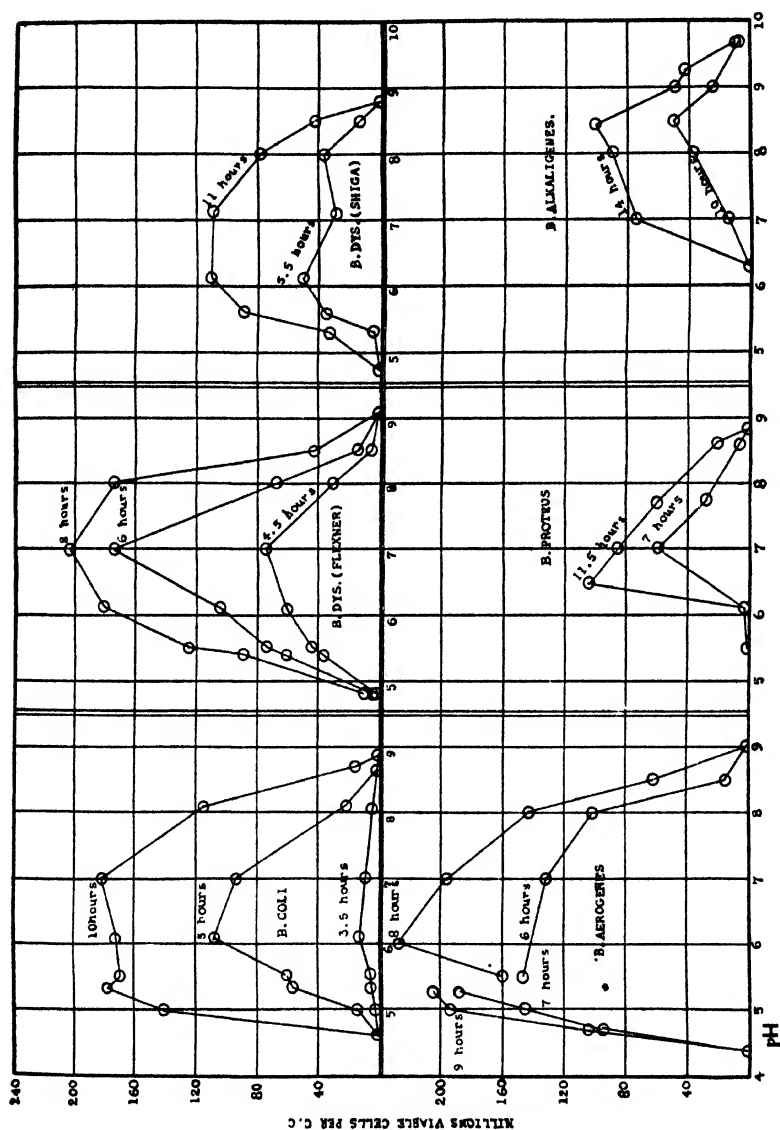


FIG. 3. CURVES SHOWING THE NUMBER OF VIABLE CELLS (ORDINATES) IN CULTURES OF DIFFERENT pH (ABSCISSAE) AT HOURS INDICATED

In other words, the period of "lag" or "latency" is very distinct and prolonged in the more alkaline media. It remains to be determined whether or not this indicates that cells transferred from a neutral to an alkaline medium require time for acclimatization.

We cannot give a complete picture of the whole course of the growth curve for our observations in most cases covered but the first ten hours, but in figure 3 are plotted a few of the measurements—sufficient to indicate the influence of pH during the early hours of incubation. Total numbers expressed as millions per cubic centimeter are plotted as ordinates and the pH values of the media as abscissae. Points for the same hour are connected to show, as it were, the rise of the wave of growth with the crest in the optimal zone. The discontinuity in the curves for *B. aerogenes* is due to the fact that two sets of experiments are represented, in one of which the original inoculum was much larger than in the other.

These curves are similar to those published by Shohl and Janney (1917) and indicate somewhat more distinct optima than do the data on maximal rates as illustrated by figure 1; but the reader should be reminded that the period covered is only the first ten hours.

As stated before, the unknown factor or factors may be predominating and may flatten these curves until the hydrogen ion effect is obscured; but even so it appears that the effect of pH is to be found in the period of lag, likewise to some extent within the period of logarithmic increase, and also in the subsequent period.

EXPERIMENTS WITH *B. BULGARICUS*

We treat these experiments separately in order to emphasize the fact that the media employed were different from those used in the other experiments. This emphasis is necessary because we wish to guard against a too literal interpretation of the comparison we shall make between the conduct of *B. bulgaricus* and *B. coli* at pH 4.5.

Multiplication studies were carried out as described before, but in a medium consisting of the extract of 10 grams dried

brewers' yeast in 1 litre of water containing 1 per cent "Difco" peptone, 0.5 per cent K_2HPO_4 , 0.2 per cent succinic acid and 1 per cent glucose. The same medium without the succinic acid but with agar was used in the plates.

Within the short and early period when logarithmic growth occurred the change in pH was not significant, but later considerable changes occurred as the sugar underwent acid fermentation. When data from these later periods are used the pH value observed at the time of the count is given.

B. bulgaricus like other organisms exhibits a remarkably abrupt change in conduct within a narrow range of pH. At pH 4.5 there was a slow but consistently steady decline in numbers during the fifteen hours of observation, while at pH 4.7 we found in one case increases during thirteen hours with a maximal rate of 1.37 generations per hour for the first period of two hours, and in a second case an initial rate of 1.21 and a slower increase in numbers during sixteen hours.

As we have already stated it is perhaps unfair to compare the conduct of *B. bulgaricus* in one medium with the conduct of *B. coli* in another of the same pH; but the fact that *B. bulgaricus* was checked at pH 4.5, when considered beside the fact that *B. coli* cultures can sometimes attain this acidity, suggests that we have here a possible explanation of why it is that under certain conditions found in the manufacture of dairy products *B. coli* gains and retains a supremacy over *B. bulgaricus*. Ordinarily *B. bulgaricus* suppresses *B. coli* for the fermentative activity of the former brings a medium well past the point endured by *B. coli*, and the particular organism with which we are now dealing can bring its culture medium to pH 3.9 (Clark, 1916). It is less severely affected at pH 3.9 than is *B. coli* slightly above its own limiting pH. The possible relation of this fact to the experiments of Gratz (1912) is suggestive.

From pH 5.1 to pH 6.7 the generations per hour average 1.6. At 6.7 a period of "lag" becomes evident. At 7.5 the lag became a period of decline five hours in length, after which there was a slow recovery in numbers. This recovery reached a maximal rate of 0.42 between the twelfth and fourteenth hours with a gradual increase in acidity to 7.1.

At pH 8.0 there was a constant decline in numbers for sixteen hours and no visible clouding of the medium at the end of twenty-four hours. Here we may call attention to the fact that several workers in their isolation of *B. bulgaricus* have successfully used media adjusted by the titration method to a zone where phenolphthalein is pink. The pH in these cases must have been as much as 8.9 or more. The discrepancy remains to be ex-

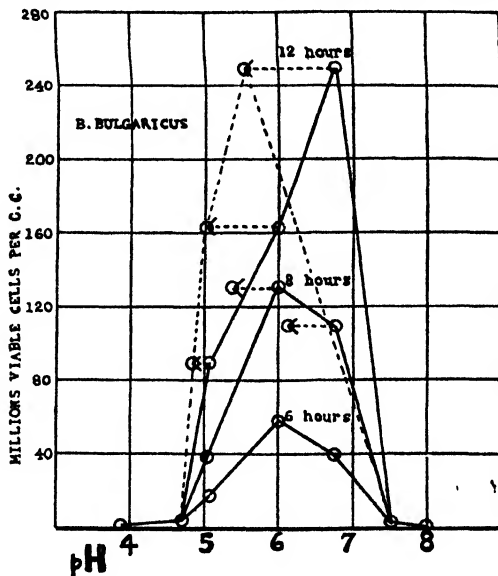


FIG. 4. CURVES SHOWING THE NUMBER OF VIABLE CELLS (ORDINATES) AT THE HOURS INDICATED IN CULTURES OF *B. BULGARICUS* STARTED AT DIFFERENT pH (ABSCISSAE)

Dotted arrows indicate shift of pH since inoculation

plained, but it may be emphasized that organisms described as *B. bulgaricus* which have come under observation in our laboratory are various in type.

A few of the counts made with *B. bulgaricus* cultures are plotted in figure 4. As will be recalled, this organism was studied in a sugar medium in order to secure satisfactory growth for our purposes. The succinic acid as buffer was effective in maintaining the desired pH with insignificant variations in most of

the cultures for six hours or longer; but when fermentation became more vigorous with the increasing numbers of the organism the pH shifted toward the acid side.

It will have occurred to the reader that we have in the data briefly outlined in the preceding pages some evidence that the effects of acidity on growth and on fermentation are independent. A striking evidence of this is to be found in the experiments with *B. bulgaricus*. Cultures of this organism were found to reach pH values consistently near 3.9 (Clark, 1916), yet growth we now find is checked at pH 4.5.

THERMAL DEATH RATES

At extreme reactions we encounter declines in the number of viable cells even at room temperature. It is obvious that we are here approaching the phase of the subject which is classified for convenience under the term thermal death rate. Long ago Pasteur (1879) in writing of the different temperatures required for the "pasteurization" of different materials said:

It is easy to show that these differences in temperature which are required to secure organic liquids from ultimate change depend exclusively upon the state of the liquids, their nature and above all upon conditions which affect their neutrality whether towards acids or bases.²

Hitherto unpublished data obtained some years ago by Clark with the assistance of Mr. Eldridge may be of interest when viewed beside the material presented in this paper.

Cultures of *B. coli* were grown eighteen hours at 30°C. in 1 per cent Witte peptone, 1 per cent glucose broth. Then to aliquot portions of this culture varying quantities of acid were added. The portions were slightly diluted with sterile water to bring each to the same volume, the pH was measured electrometrically and 5 cc. samples were placed in thin-walled vessels in a 45°C. water-bath which was vigorously stirred. In table 3 are given the amounts of hydrochloric, acetic and formic acids added to

² It is interesting to note the exact language used by Pasteur in the last phrase.

100 cc. of a culture, the resulting pH, and the time and logarithm of the counts.

TABLE 3

Influence of pH and different acids on the number of viable B. coli surviving a temperature of 45°C. for different lengths of time

N/1 HYDROCHLORIC ACID				N/1 ACETIC ACID				N/1 FORMIC ACID			
Cubic centimeters	pH	Time	Log number	Cubic centimeters	pH	Time	Log number	Cubic centimeters	pH	Time	Log number
		hours				hours				hours	
0.0	5.16	0	8 55	0.0	5 61	0	8 22	0.0	5.99	0	7.80
		1	7.83			1	8 32			1	7 99
		4	8 02			4	7 97			4	8.22
0.5	4 43	0	8 55	1.5	4.44	0	8 22	1.0	4 03	0	7 80
		1	8.13			1	7.70			1	4.08
		4	7.76			4	6.72			4	2.00
1.5	3.33	0	8 55	7.5	3 79	0	8.22	2.0	3 75	0	7.80
		1	5.45			1	4 24			1	2 00
		4	3.45			4	0.00			4	0 00
2.0	2.79	0	8.55					3.0	3.50	0	7.80
		1	2.60							1	0 00
		4	0.00							4	0.00
2.5	2.37	0	5.55								
		1	0 00								
		4	0.00								

The nature and plan of this series of experiments recall in a general way the work done by Winslow and Lochridge (1906) upon the disinfecting action of certain mineral and organic acids upon colon and typhoid organisms. The essential difference is in the fact that these authors maintained a temperature of 18°C. while the results here reported were observed at 45°C. Winslow and Lochridge noted the marked toxic effect of acetic acid—an effect much greater than that due to the hydrogen ion concentration of the acid. They explained this as being due to the undissociated acid remaining in the solution. It is however not improbable that the free acetate radicle may exert a synergic

effect upon the disinfecting power of the hydrogen ion. A positive determination of such a suggested synergic action may add appreciably to our knowledge of the mechanism of disinfection. Perhaps the difference corresponds to a difference in the permeabilities of the mineral and organic acids.

The conditions of these earlier experiments are obviously such that direct comparison with the present experiments can not be made, but the results indicate the justice of a remark by Clark and Lubs (1917a) based upon this data and the experience of Pasteur: “. . . . in cellular destruction temperature is to be considered as an accelerating *condition* among the active *agents* concerned the concentration of the hydrogen ions may be of great significance.” Buchanan (1917) has recently studied this problem in relation to canning. It is a question of another order whether the coefficients of destruction at higher temperatures become such that there is an *apparent* thermal death *point* useful when considered as such in the pasteurization of milk or other liquids. At the low temperatures of the cheese-curing room, the broader point of view is the more practical. It is also clear that in the practical application of these considerations, as for instance in canning, the variation of the effects observed with different acids in media of the same pH must be kept in mind.

SUMMARY

Although various factors, some of which are evidently quite unknown, may obscure the effect of the hydrogen ion, our preliminary study under the limited set of conditions prevailing indicates that:

1. There is a broad zone of pH within which the rates of growth are quite uniform for those short periods during which the increase of viable cells approaches the logarithmic rate.
2. On the borders of these zones of pH slight change in the pH produces a marked effect upon reproduction.
3. The acid border shifts with the nature of the acid. A distinct difference between the effect of hydrochloric and that of acetic acid was noted.

4. While no distinct optimum reaction was found when only the logarithmic increase was considered, more distinct optima appear when longer periods of growth are observed.

5. The period of "lag" is more pronounced in alkaline than in acid media.

6. Evidence is presented which suggests that the effect of pH upon specific fermentative processes, upon reproduction in its several stages and upon rates of death must be kept distinct.

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STUDIES ON THE CLASSIFICATION OF THE COLON-TYPHOID GROUP OF BACTERIA WITH SPECIAL REFERENCE TO THEIR FERMENTATIVE REACTIONS

C.-E. A. WINSLOW, I. J. KLIGLER AND W. ROTHBERG

*Department of Public Health, American Museum of Natural History,
New York*

Received for publication, October 26, 1917; literature revised
to March, 1919

INTRODUCTION

In recognition of the urgent need of progress along the lines of bacterial classification, the Society of American Bacteriologists at its meeting in Urbana appointed a special Committee on the Characterization and Classification of Bacterial Types. This Committee conceived its first duty to be a general revision of the families and genera of the Bacteriaceae, which was presented to the Society in preliminary form in 1917. In addition to this broad survey of the entire field the Committee projected a more intensive study of the colon-typhoid group, a group which, together with certain sharply defined species, includes many puzzling intermediate forms, difficult of classification, and yet of fundamental medical and sanitary importance. War work and other demands upon the time of the Committee have delayed the completion of this latter task, but certain members of the Committee, particularly Dr. Charles Krumwiede and Dr. L. A. Rogers have already published important individual contributions to the subject (Krumwiede, Pratt and Kohn, 1916a, 1916b, 1917; Krumwiede, Kohn and Valentine, 1918; Rogers, Clark and Evans, 1914, 1915; Rogers, Clark and Lubs, 1918). The present paper is the result of a detailed study of 160 cultures of colon-typhoid bacteria in the collection of the American

Museum of Natural History, conducted in large measure by the application of a series of standardized tests agreed upon by the Committee on Characterization and Classification of Bacterial Types. The laboratory work was for the most part carried out in 1915, 1916 and 1917 and preliminary reports upon its progress were made before the Society of American Bacteriologists in 1915 and 1916 (Winslow and Kligler, 1916; Winslow, Kligler and Rothberg, 1917). Final publication of the results has been delayed until time was available for a careful digestion of the data and a review of the literature concerning the classification of this group of bacteria.

THE MAJOR SUBDIVISIONS OF THE COLON-TYPHOID GROUP

Primary division on the basis of lactose fermentation. It seems reasonably certain that the typhoid bacillus was recognized in stained preparations by Eberth in 1880 and by Koch in the same year, and that Friedlaender saw *Bacterium pneumoniae* in 1882. General interest in this group of organisms dates however from the cultivation of the typhoid bacillus by Gaffky in 1884. This author described the organism as a motile rod, taking up anilin stains less readily than most bacteria, which failed to liquefy gelatin and gave a characteristic growth on potato and blood serum. Either from the presence of contaminating organisms, or a misinterpretation of irregular staining, he reported that it formed spores upon potato at 37°C.

In the following year Escherich (1885) described and figured *Bacterium coli* as a Gram-negative organism producing characteristic colonies and coagulating milk and forming gas in glucose broth. For the next decade attention was largely devoted to the establishment of distinctions between the types described respectively by Gaffky and by Escherich.

This differentiation was naturally based first of all on the production of gas in glucose media (facilitated by Smith's introduction of the fermentation tube in 1889) and on the formation of acid in lactose media. Petruschky (1889-1890) was probably the first investigator to introduce quantitative measurements of

acid production. He cultivated his bacteria in litmus milk and determined the amount of alkali necessary to produce neutrality. *B. suispestifer* gave an alkaline reaction, while the acidity produced by the other organisms studied was as follows: *B. typhosus* 0.2 to 0.3 per cent; *B. pneumoniae*, 0.3 to 0.4 per cent; *B. neapolitanus*, 0.7 to 0.8 per cent; *B. acidi-lactici*, 1.7 to 1.8 per cent. Chantemesse and Widal (1887) and Smith (1890) also pointed out that *B. typhosus* could be distinguished from *B. coli* by its less active fermentation of lactose. Chantemesse and Widal (1891), in controverting the claim of certain workers that *B. coli* and *B. typhosus* were identical, laid great stress on fermentation of sugars as a differential test and suggested the use of sugar broth containing calcium carbonate. They state that *B. coli* forms gas from glucose, sucrose, lactose, maltose, rhamnose, glycerol, erythritol and mannitol, but not from starch or glycogen; and they maintain that *B. typhosus* forms gas from none of these substances. Wurtz (1892) recommended the addition of an indicator to liquid or solid media for the easy separation of the two forms. Germano and Maurea (1893) considered the presence or absence of gas in 2 per cent glucose agar as the one absolutely diagnostic test.

In 1896 Capaldi and Proskauer suggested the use of two special media for the differentiation of the typhoid and colon groups. In medium I, containing 0.2 per cent asparagin and 0.2 per cent mannitol with the necessary salts, *B. typhosus* fails to grow and *B. coli* forms acid, while in medium II, containing 2.0 per cent peptone and 0.1 per cent mannitol, *B. typhosus* forms acid and *B. coli* does not. Medium I of course depends for its differential action on the inability of *B. typhosus* to utilize asparagin as a source of nitrogen, while the difference in the behavior of *B. coli* in the two solutions is presumably related to the balance of nitrogenous and carbohydrate foodstuffs provided. Durham (1901) urged the value of litmus whey as a test medium. Drigalski and Conradi (1902) reported that *B. coli* fermented arabinose and rhamnose while *B. typhosus* failed to do so; and they suggested a new medium for the differentiation of colon and typhoid colonies which, like the ordinary

lactose agar and the Endo (1904) medium, depends on the power of the former bacterium to produce acid from lactose. Meillère (1907) claimed that *B. typhosus* will attack inosite under aerobic conditions, while *B. coli* cannot ferment this substance.

Meanwhile many other distinctions between the typhoid and colon organisms had been described by various observers. Fremlin in 1893 differentiated *B. coli* from *B. typhosus* by its less active motility, fewer and less easily stained flagella, less tendency to occur in chains, more rapid and vigorous growth on various media (including the classical visible yellowish growth on potato), and the power to produce indol—as well as by the coagulation of milk and the formation of gas in glucose media. The active motility of the typhoid organism has since been made a basis for the selective media of Hiss (1902) and Hesse (Jackson and Melia, 1909), and for the isolation methods of Drigalski (1906) and Starkey (1906).

A new differential method of great importance was introduced in 1896 in the agglutination tests of Pfeiffer and Kolle and of Widal; and this test, too, quickly demonstrated the fundamental differences between the colon and typhoid organisms.

The more vigorous reducing power of the colon bacillus is another characteristic, which has been widely used in the form of the nitrate test. The greater ability of the colon bacillus to reduce dyes was suggested as a basis of differentiation by Dunbar (1892) using litmus, and by Rothberger (1898) using neutral red. Lead acetate on the other hand is turned brown by *B. typhosus* and not by *B. coli*, as pointed out by Orlowski, Saquépée and Chevrel (1905) and Burnet and Weissenbach (1915).

Finally the attempt to devise methods for the isolation of the typhoid bacillus has led to the recognition of a number of more or less specific differences in the tolerance of *B. typhosus* and *B. coli* toward a wide variety of antiseptic substances. The acid broths used for preliminary enrichment in the attempt to isolate the typhoid bacillus from water were designed merely to inhibit the ordinary water bacteria; but Hankin (1899) pointed out that within the colon-typhoid group *B. coli* could bear a

higher concentration of acid than *B. typhosus*. Winslow and Lochridge (1902) showed that the toxic action of the mineral acids was due to their free hydrogen ions and presented the following table of the comparative resistance of the colon and typhoid organisms.

Disinfectant action of mineral acids

	PARTS OF DISSOCIATED HYDROGEN PER MILLION NECESSARY TO EFFECT			
	99 per cent reduction		100 per cent reduction	
	HCl	H ₂ SO ₄	HCl	H ₂ SO ₄
<i>B. typhosus</i>	2.94	2.54	4.85	3.90
<i>B. coli</i>	7.49	7.68	12.80	12.60

Loeffler (1903, 1906) and Lentz and Tietz (1903, 1905) reported that *B. typhosus* was more resistant than *B. coli* to the action of malachite green. A whole group of green dyes seem to be favorable for the selective cultivation of the typhoid bacillus and its allies. Werbitzki employed China green for this purpose and Conradi, brilliant green. The whole group of the green dyes has been studied very carefully by Krumwiede and Pratt (1914). They found brilliant green most satisfactory for this purpose, the paratyphoid-enteritidis forms being most resistant, *B. typhosus* coming next, and the colon-aerogenes group being most readily inhibited. Roth (1903) and Hoffman and Ficker (1904) maintained that *B. typhosus* was more resistant to caffeine than the colon bacillus. Nowack (1905) thought that a slightly alkaline reaction exerted a selective influence on the typhoid organism. Jackson and Melia (1909) found *B. typhosus* more resistant to the action of bile salts than *B. coli*.

Davis (1914) has studied the resistance of the members of the colon-typhoid group to potassium tellurite, and finds that the colon-aerogenes forms are most resistant, followed in succession by the paratyphoid B and Gaertner group, *B. typhosus*, *B. paratyphosus* A and the dysenteries. Capsulated organisms vary widely in susceptibility, *B. capsulatus* appearing at the top and *B. pneumoniae* and *B. rhinoscleromatis* nearly at the bottom of

the list. *B. acidi-lactici* somewhat surprisingly proves one of the sensitive organisms, while *B. zopfii* is most susceptible of all.

Manfredi (1917) reports that a concentration of cholesterin of 0.5 per cent will check typhoid and the paratyphoids, while *B. coli* will grow in concentrations up to 1 per cent.

During the decade following the first cultivation of the typhoid and colon bacilli it was made clear that these organisms belong to a large group of Gram-negative non-spore-forming bacilli. They are plump straight rods with rounded ends which grow under both aerobic and anaerobic conditions on ordinary media producing colonies which vary from a thin translucent irregularly notched "grape-leaf" form to a more compact and opaque, very slightly yellowish, colony, and possessing more or less marked powers of decomposing carbohydrate materials.¹

This main group may be sharply split into two primary subdivisions by the presence or absence of the power of fermenting lactose. The lactose fermenters are generally more vigorous in their attack on other carbohydrates as well. They are less actively motile. They grow more abundantly on media and exert a more powerful reducing action. They generally produce indol. They are as a rule of low pathogenic power. The non-lactose-fermenters differ in all of the characteristics cited; and each type has its specific agglutinative reactions and its more or less specific degree of tolerance of various antiseptic substances.

SECONDARY SUBDIVISION OF THE NON-LACTOSE FERMENTERS

Considering next the further subdivision of the typhoid or non-lactose-fermenting series, the first important step was the discovery that certain members of this series possessed the power of forming gas in glucose media. *B. suispestifer* discovered

¹ Our whole conception of the morphology of this group of organisms will be radically altered if the studies of Hort (1917) are confirmed. This investigator describes in acid broth (and to some extent in ordinary broth) cultures of typhoid, paratyphoid, dysentery and colon organisms extraordinary reproductive stages, characterized by budding, terminal, lateral, or superficial, with the production of cruciform and radial cell aggregates, and he believes that detached buds may constitute a filterable stage in the life cycle of the organisms.

by Salmon and Smith in 1885 and *B. enteritidis*, demonstrated as the cause of an outbreak of meat poisoning by Gaertner in 1888, were the first organisms of this type to attract attention. Smith (1893a) pointed out that *B. suipestifer* produced gas in glucose but not in lactose media, and Durham (1898) noted that the Gaertner bacillus differs from the typhoid bacillus, not only in its power to form gas but in the lower final acidity in lactose media. The table below presented by him is an exceedingly interesting one as a product of a period when quantitative methods were so rarely used in bacteriological investigations.

Acidity, per cent normal acid

MEDIUM	PERIOD (AT 37°C.)	B. TYPHOSUS	B. ENTERI- TIDIS	B. COLI
Neutral	24 hours	0.25—0.35	0.2—0.3	0.6—0.8
Litmus whey	4-5 days	Less than 0.6	—0.15	Over 1
Litmus medium	24 hours	0.15	0.3	0.5
Litmus medium containing 2 per cent peptone	Several days	0.6	0.35	—0.2
Litmus medium containing 1 per cent glucose	Eventually	Generally alkaline	Alkaline	Alkaline

In a later communication Durham (1901) shows that litmus whey is of special value for differentiating these forms, *B. enteritidis* producing like *B. typhosus* an initial acid reaction, but unlike *B. typhosus* causing a subsequent change to alkalinity.

Meanwhile other types of colon-typhoid intermediates were being reported from various sources. Gwyn in 1898 described a case of a disease clinically like typhoid fever from which he isolated an organism resembling *B. enteritidis*, which he called a "paracolon" form (using a term first introduced by Gilbert in 1895). Cushing (1900) described a similar organism, which he called Bacillus 0, and like Durham clearly recognized the existence of a series of intermediate forms, resembling *B. typhosus* in morphology and motility, in the possession of pathogenic power, in failing to ferment lactose or produce indol, and in

relatively meager growth, but resembling *B. coli* in producing gas in glucose and differing from both in producing a transient acid reaction in milk with later reversion to alkalinity.

In 1901 Schottmüller published his classical paper on the paratyphoids. Six different strains of intermediates were worked out in detail. It was shown that these forms like those studied by Durham, Gwyn and Cushing were characterized by active motility, growth intermediate in luxuriance between that of *B. coli* and that of *B. typhosus*, gas production in glucose media, and reduction of neutral red. From the typhoid bacillus they were also distinguished by colony structure on Piorkowski's alkaline urea-gelatin, by the decolorization of an indigo compound and by a peculiar transparency in milk due to alkali production. In litmus-whey he distinguished two types of the "intermediates," one maintaining an acid reaction, the other changing to a blue-violet color. These forms, which we now know as the paratyphoids A and B, were also differentiated by their growth on media and their agglutination reactions.

By the early nineties then the non-lactose-fermenters had been separated into two clearly defined subgroups which may most conveniently be distinguished by the presence or absence of the power to produce gas in glucose media.

FURTHER SUBDIVISION OF THE NON-LACTOSE FERMENTING ORGANISMS

Each of the two groups, to which reference has been made above, was further subdivided during the early years of the twentieth century.

The first step in the differentiation of types among the bacilli which fail to form gas in either glucose or lactose was the discovery of the bacillus of dysentery by Shiga in 1898. Similar organisms were isolated by Kruse in 1900 and by Flexner and by Strong and Musgrave in the same year. Flexner (1901) distinguished the dysentery from the typhoid organisms by less active motility, general tendency to produce indol, secondary alkaline reaction in milk, and characteristic agglutination reac-

tions. After a long controversy as to the specificity of the various dysentery organisms the investigations of Martine and Lentz (1902), Duval and Bassett (1902), Hiss and Russell (1903), Park, Collins and Goodwin (1903) and others made it clear that there were at least two quite distinct types of dysentery bacilli, the Shiga type which ferments only the monosaccharides and forms no indol and a second type including the Flexner, Strong and Liiss strains which produces indol and ferments both glucose and mannitol and in some cases other carbohydrates as well. The Flexner types are most nearly allied by their fermentative reactions to *B. typhosus*, while the Shiga strains represent a group of exceedingly limited metabolic powers. J. H. Smith (1915) is, we believe, correct in using the fermentation of mannitol for the primary classification of the forms which produce no gas in glucose, and we shall follow him in recognizing as the first two subdivisions of the colon-typhoid series:

Group I. Organisms fermenting no carbohydrates except the simple hexoses (including here for convenience *B. alcaligenes* as well as the Shiga type of dysentery).

Group II. Organisms fermenting the hexoses and mannitol and certain other carbohydrates; forming only acid but no gas (including *B. typhosus* and the Flexner and other mannitol fermenting types of dysentery bacilli).

Turning now to the paratyphoids themselves, the organisms which ferment glucose with gas production but fail to attack lactose, it will be remembered that Schottmüller even in 1900 recognized two distinct types of paratyphoid organisms according to their reaction in litmus milk. Kayser (1904) emphasized this distinction more clearly and showed that Paratyphoid B resembled most closely the organisms of the Gaertner food poisoning group. Morgan (1905) studied 21 B strains and 10 A strains, the former producing a permanent acid reaction in milk and forming indol, while the latter turned the milk first acid and then alkaline, failed to form indol and gave agglutinative reactions with sera of the *B. enteritidis* group. Boycott (1906) was perhaps the first investigator to point out that even with the A strains milk cultures eventually became alkaline. This

conclusion was confirmed by Bradley (1912), Krumwiede, Pratt and Kohn (1916b), Hadley (1917) and others, and Jordan (1918) shows that the difference between the two types is probably due merely to a difference in their rate of multiplication. Buxton (1902), Bainbridge (1909) and others emphasize the less vigorous action of the A strains in glucose media, and point out that with neutral red these organisms often fail to show reduction, which is always present with the B strains. Buxton noted the peculiar opalescence in milk cultures of the B organisms and attributed it to a solution of casein caused either by the alkalinity or by the presence of a casease.

Aside from the quantitative difference in their action upon milk, the A and B divisions of the paratyphoid series exhibit other distinct differences in fermentative power. Ford (1905) concluded that *B. suispestifer* fermented only rhamnose, mannitol, and dulcitol besides the hexoses, while Schottmüller's paratyphoid A fermented arabinose also and *B. enteritidis* arabinose and xylose. Later investigations indicate that *B. suispestifer* does attack xylose and that dulcitol fermentation is generally absent in the A paratyphoids and *B. suispestifer*. The difference in dulcitol fermentation was noted particularly by Ditthorn (1913). The fact that *B. suispestifer* as well as paratyphoid B and *B. enteritidis* all ferment xylose, while paratyphoid A does not, was clearly shown by Harding and Ostenburg (1912) and Krumwiede, Pratt, and Kohn (1916a). Weiss and Rice (1917) and Hulton-Frankel (1918) point out that fermentation of inosite furnishes another differential test, paratyphoid B attacking this substance while paratyphoid A fails to do so. *B. suispestifer*, and curiously enough *B. enteritidis*, also resemble the A strains in this respect. Exhaustive studies by Krumwiede, Pratt and Kohn (1917), Jordan (1917), and Krumwiede, Kohn and Valentine (1918) have confirmed the fundamental fact that the paratyphoid group may be subdivided in the following manner on the basis of fermentative power.

	XYLOSE	ARABINOSE	DULCITOL	INOSITE
Para A	—	+	±	—
<i>B. supestifer</i>	+	—	±	—
<i>B. enteritidis</i>	+	+	+	—
Para B	+	+	+	+

Another interesting line of differentiation between the two main subdivisions of the paratyphoid group is the color reaction in media containing lead or iron salts, first suggested by Orłowski. Sacquépée and Chevrel (1905) pointed out that on a gelatin medium containing the double tartrate of iron and potassium, or subacetate of lead, paratyphoid B and *B. typhosus* produce a black coloration (due presumably to precipitation of lead sulphide), while paratyphoid A and *B. coli* fail to do so. Recently Burnet and Weissenbach (1915), Weissenbach (1917), Kligler (1917), and Jordan and Victorson (1917) have confirmed these results, which occur with marked regularity, and the latter investigators show that, as in most respects, *B. supestifer* behaves like paratyphoid A.

A similar color reaction, suggested by Orłowski, and used by Sacquépée and Chevrel (1905) and Weissenbach (1917), is the production of a green coloration in gelatin containing nitroprussiate of sodium. In such a medium paratyphoid B produces an intense green coloration in forty-eight hours and *B. typhosus* in three days; while the reaction fails entirely with *B. coli* and paratyphoid A.

The various investigations cited make it clear that we may recognize at least two more groups of the non-lactose-fermenters (in addition to those cited on p. 437).

Group III. Organisms fermenting glucose and the other hexoses with the formation of gas; fermenting mannitol and rhamnose and arabinose, in a similar way, but failing to ferment xylose and lactose and usually failing to ferment dulcitol; producing an acid reaction in milk which persists for from five days to six weeks; this group includes the A paratyphoids.

Group IV. Organisms differing from members of group III in fermenting xylose and generally dulcitol; sometimes failing to attack arabinose; producing a transient acid reaction in milk which turns to alkaline in two to five days; this group includes *B. suipestifer*, *B. enteritidis*, paratyphoid B and their allies.

SECONDARY SUBDIVISION OF THE LACTOSE-FERMENTERS

The non-lactose-fermenters may be separated, as indicated above, into four secondary groups which are rather clearly differentiated in each case by definitely correlated bio-chemical properties, as well as characteristic serological and pathogenic relations. The classification of the lactose-fermenting division of the colon-typhoid series has proved a much less simple task.

Escherich (1885) recognized two distinct types of organism in this group, *B. coli-commune* which he described as a Gram-negative rod producing characteristic colonies, gas in glucose media, and slow coagulation of milk, and *B. lactis-aerogenes* which he differentiated by the plumper form of the cells, lack of motility, and more rapid coagulation of milk. These differences seem somewhat slight but they have been shown by later observers to be correlated with much more fundamental characters. Smith (1893a) pointed out the heavier growth and tendency to capsule and zooglea formation on the part of *B. aerogenes*. In a later paper (Smith 1895a) he emphasizes the rapid gas production and relatively low acidity characteristic of this organism, and points out that the ratio of CO₂ to H₂ is higher in some strains of *B. aerogenes* (and in all the strains of *B. cloacae* studied) than is the case with *B. coli*. Russell and Bassett (1899) also laid stress on the difference in the CO₂ to H₂ ratio and suggested that the forms producing the larger proportion of CO₂ were not of intestinal origin, but were perhaps normal soil bacteria. Durham (1901) noted that the *B. aerogenes* types fermented starch and inulin and gave the peculiar reaction described by Voges and Proskauer in 1898. Grimbert and Legros in 1900 and Jordan (1903) pointed out that organisms of the *B. aerogenes* series frequently fail to form indol. A most important step in the

classification of this group was taken when Harden and Walpole (1905), and Rogers, Clark and Davis (1914) accurately measured the ratio of CO_2 to H_2 (which had been roughly estimated in an open fermentation tube by Smith and the other workers cited above); and showed that the colon forms actually produce these two gases in approximately equal proportions, while with the aerogenes type the true ratio of CO_2 to H_2 is about 2 to 1. Clark and Lubs (1915) discovered that the two types may be easily distinguished by a difference in the acid reaction produced in sugar broths of specified composition, and introduced the simple methyl-red test for measuring this difference. These investigators and others to be cited later have made it clear that in a number of respects the two types mentioned are sharply differentiated, so that we may recognize two more distinct groups of the colon-typhoid series, characterized as follows:

Group V. Organisms fermenting the hexoses, mannitol, xylose, rhamnose, arabinose and lactose, frequently salicin, dulcitol, sucrose, raffinose and glycerol; producing a strong acid reaction in media containing these substances and a moderate amount of gas, composed in equal parts of CO_2 and H_2 ; producing indol and failing to give the Voges-Proskauer reaction; generally motile and not capsulated; never liquefying gelatin.

Group VI. Organisms differing from Group V in producing a lower acidity in carbohydrate media and a larger proportion of gas, of which two thirds is CO_2 ; in fermenting dulcitol and glycerol less frequently and adonitol, salicin, starch, sucrose, raffinose and inositol more frequently; in giving a generally negative indol and a constantly positive Voges-Proskauer reaction; in being generally non-motile and frequently capsulated; and in sometimes liquefying gelatin.

The distinction between these two groups is a very sharp and clean cut one, as indicated by the large number of correlated characters in which they differ. Lines of demarcation within these groups of the lactose-fermenting organisms are unfortunately much less clear. Smith as early as 1893 recognized that among the low ratio (*B. coli*) strains there are some which attack sucrose and some which do not, and that among the high ratio forms *B. cloacae* (as described by Jordan in 1890) has the

power of effecting a sluggish liquefaction of gelatin. Durham in 1901 limited the term *B. coli-communis* to the sucrose-negative form and named the sucrose-positive form *B. communior*. Winslow and Walker (1907) pointed out that the fermentation of raffinose varies together with that of sucrose; but this is perhaps less a correlated character than an independent measurement of the same character since these two sugars have a similar molecular configuration. As a matter of fact no very striking correlations are apparent between any of the characteristics of these organisms, and the attempts made by MacConkey (1905) Jackson (1911) and others to classify them according to their fermentative reactions are purely arbitrary and artificial. The same thing is true of classifications like those of Ford (1903) and Jordan (1903) in which the liquefaction of gelatin is given an important place. MacConkey (1905) laid special stress upon dulcitol for differential purposes, Kligler (1914) upon salicin and glycerol, while Levine (1917) has made perhaps the most important contribution to the whole problem. Certain types recognized by Levine will be discussed later on, but for the purposes of a general subdivision of the colon-typhoid series it is perhaps only necessary to point out that group V as defined above includes a sucrose-positive and a sucrose-negative subgroup, while group VI includes forms which liquefy gelatin and others which fail to do so.

THE VALUE OF CARBOHYDRATE FERMENTATION TESTS AS A BASIS FOR BACTERIAL CLASSIFICATION

It will be noted that almost all observers who have attempted to differentiate and classify the organisms of the colon-typhoid series have laid primary stress upon the fermentative reactions of these organisms in carbohydrate media, and it is important to know whether such reactions are of sufficiently fundamental biological importance to warrant such an emphasis.

The results obtained by certain investigators have tended to cast some doubt upon the constancy and reliability of fermentation tests as applied to the members of this group. In the first place intermediate forms have been described which differ from typical ones either in exhibiting a slow but ultimately distinct

action upon a particular carbohydrate, or in displaying a combination of fermentative characters the reverse of that which normally occurs. Thus Wilson (1910) describes a curious form isolated from the urine of a carrier which at 37° produced no acid in lactose media and but little gas in mannitol and maltose, while at 20° it formed an abundance of acid in lactose and an abundance of gas in mannitol, maltose and salicin media. Glucose and sucrose always showed acid but no gas. Oette (1913) notes a peculiar strain which possessed the general fermentative reactions of the paratyphoids and showed specific agglutination with paratyphoid B but produced no gas. Raubitschek and Natonek (1913) isolated 31 different strains of typhoid bacilli from the various organs of two patients and found marked quantitative differences in fermentative power between them. Tenbroeck (1916) describes a variant arising in an old culture of *B. suispestifer* which retained all its normal cultural and immunological characteristics except that it no longer produced gas in glucose and failed to reduce neutral red. Smirnow (1916) exposed a series of colon strains to the influence of strong glucose solutions (3 per cent), NaCl (4 per cent), Na₂SO₄ (1.5 per cent) and phenol (0.25 to 0.75 per cent). After successive transfers, covering a period of one to three months, he found a marked suppression of biochemical activities, glucose and phenol exhibiting the most marked effects. Indol formation was first affected, then gas production in various carbohydrate media, then the characteristic growth on potato, then the coagulation of milk, and finally the production of acid in carbohydrate media. The cultures generally reverted to their normal characteristics on prolonged cultivation in ordinary media, but some of the induced modifications proved permanent. Hadley, Caldwell, Elkins and Lambert (1917) recognize two varieties of *B. pullorum*, one of which produces gas while the other fails to do so. Oliver and Perkins (1918) report the case of a dysentery-like organism which under ordinary conditions attacked glucose only but which when cultivated under diminished oxygen tension acidified galactose, maltose, levulose, mannitol, lactose and sucrose. It is very probable, as shown by recent work, that results were complicated by the buffer action of CO₂. Bron-

fenbrenner and Davis (1918) have isolated a number of organisms which when first studied fermented lactose very slowly, but whose power to attack this sugar could be progressively increased by cultivation in lactose media.

Such differences as those described may be in large measure explained as the result of a direct response to different environmental conditions, or as the normal quantitative variations exhibited by all biological reactions. In using any biochemical characteristic for differential purposes it is necessary to specify a standard set of conditions and to use modal points in the curve of distribution of quantitative values, rather than arbitrary limits, in defining groups. In connection with some of the irregularities observed it must be remembered that the complex sugars used by bacteriologists frequently prove on careful analysis to contain hexose-impurities; and that if not originally present the hexoses may be produced by excessive autoclaving. Another type of investigation indicates however that the causes of observed variations may be of a more deep seated nature. Twort (1907) studied the action of 18 colon-typhoid organisms on 49 different glucosides, and on the basis of the results obtained concluded that no significant differences existed and that all the organisms studied were "varieties or hybrids of one or more species." He then tried to produce modifications of fermentative power by cultivating various strains for long periods in media containing carbohydrates which they would not at first utilize, and found that they slowly acquired new fermentative powers when treated in this way. All the members of the paratyphoid group acquired the power to ferment sucrose, *B. typhosus* began to ferment dulcitol and lactose, the dysentery bacilli of Kruse and Flexner were able to ferment sucrose within twenty-four hours. Neisser (1906), Massini (1907), Burk (1908), Mueller (1908, 1909), and Penfold (1910, 1911a, 1911b, 1912) have described what appear to be clear-cut and definite cases of mutation in this group of organisms. The phenomena recorded are in general as follows: an organism cultivated on a solid medium containing a carbohydrate, which it normally fails to attack, produces colonies which at first appear (as indicated by the reaction of the indicator contained in the medium) as of the

usual non-fermenting type. Later however papillary projections appear on the colony, which are red if litmus be present, and plates made from these papillae yield a certain proportion of colonies which from the first exhibit vigorous fermentative power. Penfold (1910) studied Twort's lactose-fermenting strain of *B. typhosus* and found that it readily threw off non-fermenting mutants. In general however he found the fermentation of lactose to be a fairly stable character but dulcitol and rhamnose exhibited the phenomenon of mutation in a large number of strains. According to Mueller (1908, 1909), of 120 strains of *B. typhosus* all produced rhamnose-fermenting mutants. Mueller and Penfold (1912) both observed raffinose-fermenting mutants in colonies of members of the paratyphoid group. Of 34 strains of non-lactose-fermenters isolated by Penfold from feces, 21 produced fermenting mutants of some sort. The relation between the phenomenon of papilla-formation and the gradual increase in fermentative power, often observed (as by Bronfenbrenner) in liquid media, is well brought out in one of Penfold's investigations (1911a). In a dulcitol broth culture of *B. typhosus* which became acid to litmus after ten days of incubation he found, by plate cultures made from day to day, that at first fermenting strains were rare but that their number suddenly increased after the first week. Successive transfers in dulcitol broth produced a mass culture which turned litmus broth red in one day, presumably as a result of progressive selection of the dulcitol-fermenting mutant. In a later paper Penfold (1911b) shows that cultivation of certain colon and paratyphoid strains on chloracetic-acid agar produces varieties which have lost all power of producing gas in sugar media, while the fermentation of the alcohols is unaffected. Revis (1911) notes a similar phenomenon, a colon organism grown in presence of 0.05 per cent malachite green losing the power of gas production and after more prolonged treatment failing to coagulate milk and to form acid in dulcitol. Ledingham (1918) reports two highly variable strains of Flexner-Y dysentery, one of which produced mutants fermenting rhamnose and arabinose, while the other normally acidified these sugars but produced a mutant which failed to do so.

A general survey of this evidence indicates that the fermentative characters of the colon-typhoid bacteria are not only influenced by environmental conditions but also exhibit inherent powers of spontaneous mutation; yet it does not materially weaken the general value of tests of fermentative ability for systematic purposes. It is significant that spontaneous mutations are most common with dulcitol and rhamnose, carbohydrates which have been shown in comparative studies to lack the correlations with other characters which are exhibited by lactose, sucrose, xylose, and other substances; and that other modifications, such as the suppression of gas production, have generally been associated with strikingly abnormal environmental conditions. Taking the great mass of colon-typhoid strains, as they are isolated from the bodies or intestines of men and animals, and cultivated under standard conditions, fermentative characters exhibit a high degree of constancy and what is even more important a high degree of correlation with other bio-chemical and serological and pathogenic properties. It is no accident that the disease-producing organisms of the colon-typhoid series are practically without exception organisms which fail to produce gas in lactose media, but a law, which can have its basis only in the principle of phylogenetic relationship; and the same principle holds for numerous correlations which have been cited in the description of the six principal subdivisions of the colon typhoid series which have been recognized in the preceding section. Even Twort's studies, when properly analyzed, exhibit clear evidence of the general relationships of the chief groups of organisms concerned and of their progressive increase in fermentative power in proceeding from one end to the other of the series. His *B. alcaligenes* strains attacked none of the glucosides studied. With dysentery and typhoid organisms 7 per cent of his glucoside tests were positive; with the paratyphoid and Gaertner forms, 15 per cent; with colon bacilli, 46 per cent; and with *B. aerogenes* types, 65 per cent. We are dealing here with a group in which the line of evolutionary development has been marked most clearly by the progressive loss or progressive acquisition of fermentative power. Intermediate types appear at times and mutants may occur; but on the whole,

the series of forms between *B. alcaligenes* at one extreme and *B. aerogenes* at the other may be broken up into six or more main groups on the basis of fermentative ability; and the groups so constituted are true biologic entities marked by a number of independent correlated characters which can only have their origin in phylogenetic relationship.

THE CHEMISTRY OF THE FERMENTATIVE REACTIONS OF THE COLON-TYPHOID GROUP

The gaseous products of carbohydrate decomposition. The production of gas is the most obvious and striking evidence of carbohydrate decomposition and ever since Smith (1890) introduced the fermentation tube into bacteriology the appearance of visible gas in the Smith or Durham tube has been used as one of the most common criteria of fermentative activity. It is obvious that such a test is but a rough and approximate one and that no sharp line can be drawn between gas-producers and non-gas-producers, since a certain amount of gas will be necessary to saturate the liquid medium in the tube before excess gas will collect and become visible in the closed arm. Clark (1913) criticised Penfold's emphasis on the non-gas-forming mutants produced by the influence of chloracetic acid on this ground, and has pointed out that since normal *B. coli* produces a smaller amount of gas in glucose and galactose media than in dulcitol and mannitol the suppression of gas formation in the former case and not in the latter is perhaps merely due to a general weakening of the organisms concerned. The method of studying carbohydrate fermentation by examining the gas which collects over a liquid, which is exposed freely to the air at another point, is still less suited for careful studies of the composition of the gases present. Smith in his classic investigations laid stress not only upon the proportion of CO₂ to H₂ (which he reported as 1:2 for *B. coli*—Smith 1890) but also upon the quantitative determination of the total amount of gas present (which he states as 40 to 70 per cent for *B. coli*, *B. proteus* giving less and *B. cloacae* more,—Smith 1893b). It seems obvious that the amount and composition of the gas collected in the Smith tube will be governed almost as much by the solubility of the gases produced, and the rate at

which they diffuse outward from the free surface of the liquid medium, as by the inherent biochemical activities of the organisms which produce them, and measurements made in the Smith or Durham tube will always be accompanied by the loss of a large and a variable proportion of the CO_2 actually produced.

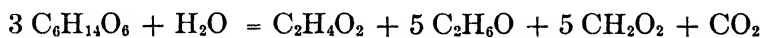
As a matter of fact, even before the introduction of the fermentation tube into bacteriology, the real composition of the gases produced in bacterial fermentation had been established by several observers who used the proper methods to secure a sample of all the gas actually produced, by cultivation in closed bulbs which were later evacuated by the air pump. As early as 1887 Hoppe-Seyler observed that calcium formate infected with river mud yielded a gas composed in equal volumes of CO_2 and H_2 . Frankland and Fox (1889) were perhaps the first investigators to work with a pure culture, using a form which they isolated from sheep dung and named *B. ethaceticus*. This organism was said to be a gelatin liquefier but curiously enough gave the fermentative reactions of *B. coli* since Frankland and Lumsden (1892) and Frankland and MacGregor (1892) report an equal volume of CO_2 and H_2 produced in glucose, mannitol, and arabinose. Macfadyen, Nencki and Sieber (1891) reported that the gas produced by *B. aerogenes* was 72 per cent CO_2 and 28 per cent H_2 . Frankland, Stanley and Frew (1891) estimated a ratio of CO_2 to H_2 of 10 to 8 for *B. pneumoniae*. Pakes and Jollyman (1901) also reported the 1:1 ratio in the decomposition of sodium formate by paratyphoid, colon and aerogenes types. By far the most exhaustive studies of this question were made, however, by Harden (1901, 1905) who placed the distinction between the 1:1 ratio for *B. coli* and the ratio of 2 or more parts of CO_2 to 1 of H_2 for *B. aerogenes* on a definite and solid basis.

In this country Bennett and Pammel (1896) showed that the gas formed in the Smith tube is at first only H_2 and that the ratio between H_2 and CO_2 depends mainly on the absorption of CO_2 by the liquid medium; and Longley and Baton (1907) made clear the slight value of measurements of the amount of gas and the ratio of its components in the open tube. Keyes (1909) and Keyes and Gillespie (1913) made the first exact studies in America of the actual gas production by exhaustion methods and

Rogers, Clark and Lubs extended and confirmed the work of Harden and broadened the basis of the radical distinction between the high and low ratio groups.

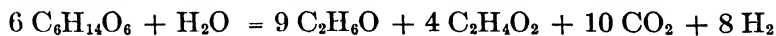
The acid products of carbohydrate decomposition. Studies of gas production have been given first place in this review of the subject because of the fact that gaseous products are most easily measured and have therefore attracted more general attention. A real comprehension of the processes involved demands however a knowledge of the soluble products as well.

In the classic studies of Frankland and his associates on *B. ethaceticus* it was shown that this organism when decomposing mannitol and glycerol produces chiefly ethyl alcohol and acetic acid, with smaller amounts of formic and succinic acids, the ratio of alcohol to acetic acid being 2 molecules to 1 in the case of mannitol and 3 to 1 in the case of glycerol. (Frankland and Fox, 1889). In later papers (Frankland and Lumsden, 1892, Frankland and MacGregor, 1892) it is stated that the proportion of acetic acid is higher with glucose than with the alcohols mentioned above, still higher with arabinose and highest of all with glyceric acid. When the fermentation takes place in a closed space a considerable amount of formic acid accumulates and a formula for the decomposition of mannitol is suggested as follows:



In the open tube it is assumed that the formic acid is promptly decomposed into H_2 and CO_2 .

With *B. pneumoniae* under the conditions of ordinary cultivation Frankland, Stanley and Frew (1891) give a slightly different formula for the decomposition of mannitol



Frankland and his associates apparently failed to lay emphasis on the non-volatile acids, although they suggest the presence of traces of a fixed acid (probably succinic). Macfadyen, Nencki and Sieber (1891) however report ethyl alcohol and acetic and lactic acids in glucose broth cultures of colon-group organisms, the proportion of acetic acid being less in the case of *B.*

aerogenes than in cultures of *B. coli*. Grimbert (1896) gives the results tabulated below for the Friedlaender bacillus and *B. coli*.

Products of permanent activity

	GRAMS FORMED PER 100 GRAMS OF CARBOHYDRATE FERMENTED					
	B. pneumoniae				B. coli	
	Mannitol	Dulcitol	Arabinose	Xylose	Glucose	Lactose
Ethyl alcohol	11.4	29.3	0.0	6.9	Trace	6.8
Acetic acid	10.6	9.6	36.1	23.4	14.3	25.4
Laevo lactic acid	36.6	0.0	49.9	Trace	42.7	Trace
Succinic acid	0.0	21.6	0.0	19.9	Trace	29.8

Duchacek (1904) found that in media exposed to air *B. coli* produced approximately equal amounts of lactic and acetic acids, but that under an atmosphere of hydrogen the proportion of lactic acid increased to 2:1. *B. typhosus* formed a considerably higher proportion of lactic acid than *B. coli*.

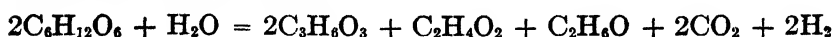
Harden's work constitutes by far the most extensive investigation into this question. Harden and Walpole (1906) give the following results of the fermentative activity of *B. coli* and *B. aerogenes*.



Products of fermentative activity

	GLUCOSE		MANNITOL	
	B. coli	B. aerogenes	B. coli	B. aerogenes
Per cent of carbohydrate fermented				
Alcohol	12.8	18.2	28.1	32.5
Acetic acid	18.8	8.6	9.5	2.1
Lactic acid	31.9	9.1	18.6	8.6
Succinic acid	5.2	4.5	8.9	2.8
Formic acid	0.0	1.7	3.0	1.6
Carbon dioxide	18.1	35.2	28.4	35.5
Cubic centimeters per gram				
CO ₂	91.8	178.5	143.0	180.3
H ₂	110.0	92.4	167.0	143.6
Ratio H-CO ₂	1.19	0.52	1.18	0.79

The results for *B. coli* with glucose would correspond roughly to the following formula:



Levulose behaves in the same way; and the general proportion of the end-products in the case of the hexoses and the alcohols is explained in the following scheme.

HEXOSE-GLUCOSE		PRODUCTS	ALCOHOL - MANNITOL		PRODUCTS
CH ₂ OH		C ₂ H ₆ O +CO ₂ +H ₂	CH ₂ OH		C ₂ H ₆ O +CO ₂ +H ₂
CHOH	CH ₂ OH		CHOH	CH ₂ OH	
-----	-----		-----	-----	
CHOH	CHOH	2C ₂ H ₆ O ₂	CHOH	CHOH	2C ₂ H ₆ O ₂
CHOH	CHOH		CHOH	CHOH	
CHOH	CHOH		CHOH	CHOH	
CHOH	CHOH		CHOH	CHOH	
-----	-----	C ₂ H ₄ O ₂ +CO	-----	-----	C ₂ H ₆ O +CO ₂ +H ₂
CHO	CHOH		CH ₂ OH	CHOH	
	CHO			CH ₂ OH	

For glucose this scheme would yield 2 molecules of lactic acid and 1 each of acetic acid and alcohol, for mannitol 2 each of lactic acid and alcohol; and to explain the observed results it is necessary to assume that in the latter case the lactic acid is largely destroyed by secondary reactions. Glycerol according to Harden is split directly into alcohol and formic acid.

In the case of *B. aerogenes* the proportion of lactic acid to alcohol formed is lower with glucose and very much lower with mannitol, while the acetic acid is even more reduced. It is evident that the portion of the carbohydrate molecule which furnishes the acids in the case of *B. coli* is here split in a different way and Harden and Walpole (1906) have shown that the end product in the *B. aerogenes* fermentation is chiefly 2:3-butylene glycol with some acetyl methyl-carbinol. The latter substance as shown by Harden (1906) and by Harden and Norris (1912) is the active agent in producing the Voges-Proskauer reaction. The acetyl methyl carbinol in the presence of potash and oxygen is oxidized to CH₃.CO.CO.CH₃ (diacetyl) and the diacetyl reacts with some constituent of the peptone medium to produce the eosin-like fluorescence characteristic of this reaction.

Harden's studies were confined for the most part to the gas-producing organisms but he points out that with forms like *B. typhosus* formic acid must be produced from the fractions of the molecule which with *B. coli* yield CO_2 and H_2 . Sera (1910a, 1910b) has investigated this point and reports that typhoid and dysentery bacilli in decomposing glucose, glycerol or mannitol produce acetic and formic acids, with some propionic acid in the case of glycerol. In glucose and glycerol acetic acid is in excess, in mannitol formic acid. *B. typhosus* also forms a trace of alcohol.

An illuminating study was made by Grey (1914) of the fermentative products of Penfold's chloracetic acid mutants, in which the power of gas production had been reduced. He found that in each case the selected strains produced in glucose media more lactic acid and less alcohol, acetic acid and formic acid, and also decomposed less of the formic acid which they did produce. In mannitol however the change was limited to a decrease in decomposition of formic acid, the primary products formed being the same. Grey assumes that the glucose molecule is normally split, first into lactic acid and an Intermediate substance A, which in turn yields formic acid and an Intermediate substance B (possibly acetaldehyde). The Intermediate substance B finally yields alcohol and acetic acid. Grey believes that the decomposition of Intermediate substance B is due to a reduction, accomplished ordinarily by the excess of H_2 present in the case of mannitol but by a special reductase in the case of glucose. If the formation of this reductase were suppressed by chloracetic acid it would explain the fact that, while Intermediate substance B undergoes a normal splitting in the case of the selected strains when acting on mannitol, the process is fundamentally affected in the case of glucose. The decomposition of formic acid is of course equally affected in both instances.

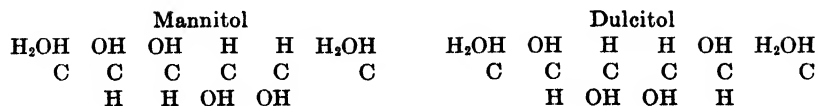
It is evident that the changes which go on in the fermentation of carbohydrate media are complex and for the most part still obscure; but the broad facts appear to be established that the process leads to the production of alcohol and of acetic, lactic, formic and succinic acids (the latter in small amount); that the

decomposition of the formic acid and perhaps of other constituents of the molecule produces a gas composed of carbon dioxid and hydrogen; that in the decomposition of mannitol alcohol is produced in much greater, and lactic acid in much smaller, amount than is the case with glucose; that in the case of *B. aerogenes* butyleneglycol, acetylmethylcarbinol and an excess of carbon dioxid are formed at the expense of the portion that with *B. coli* yields lactic and acetic acids.

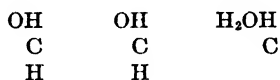
Relation between the structural formula of the carbohydrates and their decomposition by colon-typhoid bacteria. The question of the relation between molecular structure and fermentability is of the greatest interest to the biologist, since data in regard to such a relationship may reasonably be expected to throw important light upon the underlying bio-chemical processes involved. The studies made upon the colon-typhoid group justify a few fundamental generalizations in regard to this point.

First of all it is evident that the hexoses are more easily attacked than any of the other substances ordinarily investigated. All of the members of the colon-typhoid group, with the exception of *B. alcaligenes*, produce a marked increase of acidity in these carbohydrate media. In our own studies we find that fructose, mannose, and galactose behave essentially like glucose, all the strains which acidify glucose acidifying all the other hexoses as well.

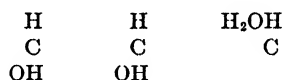
The hexahydric alcohol, mannitol, is the substance which comes next in order of availability, all of the groups studied except *B. alcaligenes* and the Shiga dysentery types acidifying this substance. Dulcitol however, which resembles mannitol so closely, is much more resistant. As a matter of fact dulcitol occupies a somewhat unique position among all the carbohydrates whose decomposition has been extensively studied. It is the only one whose fermentation seems wholly uncorrelated with that of other carbohydrate media. In all the principal groups of the paratyphoid, colon and aerogenes series we find forms which ferment this alcohol and others which fail to do so; and it has been pointed out that this particular fermentation is the one which exhibits the most general tendency toward mutative variability.



Sorbitol appears to behave like mannitol, being attacked by all the paratyphoid types according to Boycott (1906), Jordan (1917), and Krumwiede Kohn, and Valentine (1918). From the formulae of these alcohols it may be noted, as suggested by Revis (1910) that the configuration of mannitol and sorbitol resembles that of the alcohol half of the hexoses. In the hexoses the grouping is



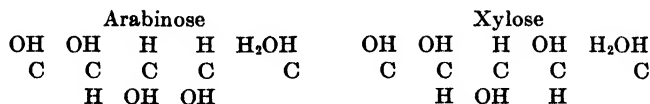
In mannitol and sorbitol it is



In dulcitol on the other hand there is no alcohol grouping with two adjacent carbon atoms having the hydroxyls on the same side; and in this difference may perhaps lie the difference in susceptibility to bacterial decomposition.

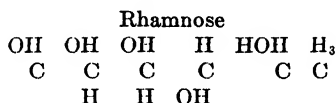
Next to these alcohols in availability for the colon-typhoid organisms comes the disaccharide maltose. We have not studied this carbohydrate ourselves but Hiss and Russell (1903), Hiss (1904), Smith (1915), and others show that the Shiga type of dysentery bacillus fails to attack maltose, while Morgan (1906, 1911) reports negative results for the peculiar organism which bears his name, as do Hadley, Elkins and Caldwell (1918) for *B. pullorum*. On the other hand Drigalski and Conradi (1902), Boycott (1906), Sacquépée and Chevrel (1905), Savage (1912), Jordan (1917), and Krumwiede, Kohn and Valentine (1918) find that the typhoid and paratyphoid organisms all attack maltose readily.

The next substances, in order of availability, appear to be xylose and arabinose with the structural formulae indicated below.



Here we may note the very interesting fact that among the less active fermenters of the typhoid and paratyphoid series (where the fermentation of these sugars first appears) action upon xylose and arabinose seems to be inversely correlated. Thus the Flexner dysentery and paratyphoid A types attack arabinose but not xylose, the typhoid and hog-cholera strains xylose but not arabinose. It would appear that the power to ferment the two different molecular groupings represented by these sugars appears or disappears independently and they are presumably attacked in a quite different way.

Rhamnose is the next carbohydrate to be attacked by the colon-typhoid bacteria.² This substance, which is a methylated pentose, is utilized by all the paratyphoid, colon and aerogenes series; and it may be assumed that the presence of the methyl group is the circumstance which prevents the Flexner dysentery and typhoid organisms from decomposing it.



From the five and six carbon carbohydrates to the disaccharide lactose there is a distinct and well marked step; and we may reasonably assume that the broad distinction between the pathogenic dysentery-typhoid-paratyphoid series on the one hand and the non-pathogenic colon-aerogenes series on the other is correlated with the absence or presence of an enzyme capable of breaking up this disaccharide into glucose and galactose. The

² In this discussion we have for convenience considered the carbohydrates in their order of availability, passing from those most easily to those least commonly fermented. It is not necessary however to assume that evolution has proceeded along the line of a progressive acquisition of fermentative properties. Since the organisms of low fermentative power are the pathogenic (and presumably more recent) forms it would be more reasonable to assume that development has been in the direction of a loss rather than a gain in fermentative power, in which case *B. aerogenes* might be the primitive ancestral type of the whole group.

difference between maltose and lactose in availability is a striking and significant one. It has been noted that all the members of the typhoid and paratyphoid series produce acid from maltose; but the power to decompose lactose appears only among the gas-producing forms of the colon and aerogenes groups.

It seems clear that the processes involved in the decomposition of the two disaccharides, maltose and lactose, must be fundamentally dissimilar; and it is of interest to note that while the difference in availability between mannitol and sorbitol and between the three pentoses are associated with differences in reactive organic groups, the even more fundamental difference between the availability of maltose and lactose can be explained only by a stereoisomeric difference in the molecules concerned. As in the case of animal enzymes the presence of maltase and lactase seems to vary quite independently.

Next to lactose in order of availability come sucrose and raffinose; and here we find another definite relation between fermentability and molecular structure. The correlation between the fermentability of these two sugars has been shown by Winslow and Walker (1907), Burk (1907), Howe (1912), Rogers, Clark and Davis (1914a), Kligler (1914), Levine (1916) Murray (1916), and Rogers, Clark and Lubs (1918) to be an almost perfect one. These two sugars, although one is a disaccharide and the other a trisaccharide, are alike in the absence of the aldehyde group; and this fact may be assumed to account for the similarity in their behavior.

The fermentation of the glucoside salicin is generally, but not always, correlated with that of lactose; being fermented by most, but not all, of the colon group and by all the *B. aerogenes* strains; while the cyclohexanhexol inosite appears to be fermented only by Paratyphoid B and certain *B. aerogenes* strains.

PROGRESSIVE CHANGES OF REACTION IN CARBOHYDRATE MEDIA AND THEIR SIGNIFICANCE

The estimation of the individual products of carbohydrate decomposition is of course a difficult and time consuming process; and even the determination of the gases evolved must be

carried out by exhaustion from a closed vessel if it is to have any serious value. The acidity produced in a given culture medium is on the other hand a characteristic which can be easily and accurately measured. To this point we have therefore devoted a considerable share of our attention.

When the work on the colon-typhoid group was begun we made our first studies of acid production by the old titration method, cultivating each strain in broth (containing 1 per cent Digestive Ferments Company peptone, 0.5 per cent K_2HPO_4 , and 1 per cent of carbohydrate) at $30^\circ C$. After two days and seven days of incubation, tubes were withdrawn, held in steam or boiling water for ten minutes to drive off CO_2 , cooled and titrated against N/20 NaOH with phenolphthalein as indicator. The general results of the observations made by this method on a series of colon-aerogenes strains have been already reported by one of us (Kligler, 1914). They indicated that in any carbohydrate medium there is a fairly sharp distinction between fermenting and non-fermenting forms, the line between the two groups being at about 1.5 per cent normal acid, (as ordinarily determined), the mode for the non-fermenters being near the neutral point and that for the fermenters varying between 2.5 and 3.5 per cent acid for the various carbohydrates.

In view of the rapidly accumulating evidence which indicated that the measurement of acidity by the titration method is inaccurate and often misleading, we determined the true acidity in the various fermentable media for our complete series by the use of the Clark and Lubs indicators. The media first used for this purpose contained 0.5 per cent Digestive Ferments Company peptone 0.5 K_2HPO_4 and 0.5 per cent of the fermentable substance to be studied, as recommended by Clark and Lubs. We found however that a somewhat sharper differentiation could be obtained by increasing the amount of sugar, and in our regular routine studies the medium was made up with 0.5 per cent peptone, 0.5 per cent K_2HPO_4 and 1 per cent of the fermentable substance. The cultures were incubated at $30^\circ C$. and the acidity was determined after two, four and five days by the use of methyl red, dibromthymolsulphonphthalein and phenol sul-

phonphthalein, according to the methods suggested by Clark and Lubs (1917).

In general four distinct types of reaction were observed when the cultures were examined in this way, reactions which we have described as types I, II, III, and IV (Winslow, Kligler and Rothberg, 1917). In type I which is observed when the carbohydrate present is not attacked at all, the reaction remains throughout in the neighborhood of P_H 7.0. Type II is the reaction characteristic of slow fermentation, the P_H value after two days being still at 6 or over, and only later reaching an acidity below 5.5. This type of reaction appears with substances which are decomposed with some difficulty, such as dulcitol, rhamnose, and salicin, and the principal results of this kind which we obtained are tabulated below.

Type II reactions

GROUP OF ORGANISMS	FERMENTABLE SUBSTANCE	AVERAGE P_H VALUES AFTER		
		2 days	4 days	5 days
Flexner dysentery.....	Mannitol	6.5	5.5	5.7
Paratyphoid A.....	Rhamnose	6.4	5.0	5.1
	Arabinose	7.0	5.0	5.1
B. suipestifer.....	Rhamnose	7.0	6.4	5.8
Paratyphoid B	Rhamnose	6.7	5.0	5.1
	Dulcitol	6.7	5.3	5.2
B. communior.....	Dulcitol	7.0	5.6	5.5
	Salicin	5.9	5.1	4.8
B. communis.....	Sucrose	6.1	5.2	5.2
	Salicin	6.4	5.4	5.3
	Dulcitol	7.1	5.6	5.5

A type III reaction is the normal reaction for the colon-typhoid organisms in any carbohydrate which is readily fermented. In this case the acidity rises rapidly, usually within twenty-four hours and always within forty-eight hours, to a P_H value of 5.3 or less and remains practically constant at about that point.

We have observed this rapid rise and subsequent maintenance of high acidity with all the groups of organisms studied (except *B. aerogenes*) and in the case of all the substances fermented except those cited above as giving a somewhat delayed reaction. With these exceptions the P_{∞} value after two days of incubation varied for all the groups studied and for all the substances fermented between the limits of 5.0 and 5.6, while the final values after five or six days varied between 4.8 and 5.3. This final reaction averaging about 5.0 is so constant that it may be fairly assumed to represent the limiting concentration of acid for the colon-typhoid organisms in the particular medium used for our studies. Clark (1915) has pointed out the same constancy in final reaction for *B. coli* in 30 different media, his range of variation (even with a series of variously buffered media) being only from P_{∞} 4.3 to P_{∞} 5.3. Michaelis and Marcora (1912) reported 4.8 as the limiting P_{∞} value for *B. coli* in glucose peptone broth. Our results make it possible to extend these conclusions and to state that not only for *B. coli* but for the whole colon-typhoid group the final acidity in sugar broths is practically the same,—about P_{∞} 5.0—in the medium studied by us.

In a longer period of incubation than that used for our routine determinations (five days) there appears to be a slight reversion toward alkalinity even in the case of the type III reaction, as indicated by the results tabulated below for a series of 9 typical typhoid strains; and this slight reversion may probably be attributed to the formation of alkaline products of protein decomposition.

Average acidity (P_H) produced after various periods by nine typhoid strains

	1 DAY	2 DAYS	4 DAYS	6 DAYS	8 DAYS	11 DAYS	13 DAYS
Glucose	5.3	4.9	5.0	5.2	5.3	5.3	5.4
Mannitol.....	5.5	5.1	5.4	5.6	5.8	5.9	5.8

It will be noted that the P_{∞} values are slightly higher throughout in the mannitol as compared with the glucose broth. Our results indicate that there exist minor differences of this kind, the fermentation of carbohydrates which are less readily utilized being a trifle slower and producing a little lower acidity than the

decomposition of glucose. The line between a type II and a type III reaction is not therefore an absolutely sharp one.

The type IV reaction, in which the acidity rarely goes below a P_H value of 5.5 and quickly reverts to a value over P_H 6 is characteristic of the *B. aerogenes* group and appears to be related to a fundamentally different action upon the fermentable substances. The differences between the type III and type IV reactions during the first forty-eight hours are indicated below for a series of *B. coli* and *B. aerogenes* strains.

Average acidity (PH) produced by a series of strains of B. coli and B. aerogenes in glucose broth

	2 HOURS	4 HOURS	8 HOURS	24 HOURS	48 HOURS
<i>B. coli</i>	7.3	7.2	6.0	4.9	4.7
<i>B. aerogenes</i> ..	7.2	6.8	5.8	5.7	6.2

The reactions cited below for *B. aerogenes* during a longer period of incubation in various carbohydrate media are for a selected series of 10 typical strains and may be compared with corresponding figures quoted above for *B. typhosus* in glucose and mannitol.

Average acidity (PH) produced by a series of B. aerogenes strains

	1 DAY	2 DAYS	4 DAYS	6 DAYS	8 DAYS	11 DAYS	13 DAYS
Glucose.....	5.3	5.3	5.8	6.2	6.3	6.6	6.3
Lactose.....	5.6	5.6	5.9	5.9	6.1	6.8	7.0
Sucrose.....	5.4	5.5	6.0	6.4	6.5	7.1	6.8
Rhamnose	5.5	5.4	5.7	5.1	5.9	6.3	6.3

In the type IV reaction, marked differences in rate of fermentation appear between individual strains and a high acidity (in some cases as high as P_H 5.1) may be attained for brief periods. The reversion is very rapid however and P_H values lower than 5.5 are rarely observed.

The phenomenon of reversion from a preliminary acid reaction to a more alkaline one was explained by the earlier workers on this subject as due to exhaustion of the carbohydrate, followed by the utilization of nitrogenous foodstuffs with the release of alkaline products of protein decomposition.

Kendall, Day and Walker (1913) made a somewhat extensive study of this point, determining the reaction of various members of the colon-typhoid group in plain broth and glucose broth by the use of neutral red as an indicator, and simultaneously estimating the rate of protein decomposition by the increase in ammonia. Their results indicated a definite sparing action of the sugar. For example *B. dysenteriae* in plain broth produced 2.1 to 4.2 mgm. of NH_3 per 100 cc. broth with no material change in reaction, while in 1 per cent glucose broth the reaction reached an acidity of 2 to 2.8 per cent Normal to neutral red, while no ammonia was formed. *B. typhosus*, the paratyphoids, the Morgan bacillus, and *B. coli* all behaved in essentially the same way; while in the case of *B. cloacae* the carbohydrate was apparently destroyed so quickly that it made little difference in the protein metabolism whether glucose was present or not. In the case of *B. alcaligenes* there was no difference in reaction between the plain broth and the glucose broth, but growth was more luxuriant and more ammonia was produced in the latter case, suggesting some sort of utilization either of the glucose itself or of impurities contained therein. The range of values recorded for certain groups are of such interest as to warrant reproduction.

ORGANISM	BROTH	REACTION TO NEUTRAL RED	INCREASE IN NH_3
<i>B. dysenteriae</i>	Plain	-0.3 to +0.2	2.1 to 4.2
	Glucose	2.0 to 2.8	None
<i>B. typhosus</i>	Plain	+0.1 to -0.9	4.2 to 10.5
	Glucose	2.9 to 4.9	None
Paratyphoids	Plain	-1.5 to +0.3	4.2 to 16.1
	Glucose	2.3 to 4.8	0.0 to 2.8
Morgan bacillus	Plain	-0.5 to -2.1	20.3 to 42.0
	Glucose	2.9 to 4.6	4.2 to 8.4
<i>B. coli</i>	Plain	-0.4 to 1.9	11.2 to 36.4
	Glucose	3.9 to 6.1	-1.4 to 2.1
<i>B. cloacae</i>	Plain	-0.9 to -1.4	20.3 to 21.7
	Glucose	-0.6 to +1.0	16.8 to 19.0

These results do indeed suggest that the difference in reaction in a carbohydrate medium may be partly due to the relative rate at which protein and carbohydrate, respectively, are being attacked. That in the case of certain organisms there is something much more fundamental involved is indicated by the peculiar behavior of *B. cloacae* (a form which belongs to the *B. aerogenes* group); and the same deep-seated difference is indicated by the progressive change of reaction in glucose broth recorded by Kendall, Day and Walker for the various groups of organisms studied. We have calculated below the average values for certain of these groups and in so doing have separated one of the groups presented by the authors under the name of *B. mucosus-capsulatus* into two subgroups since 9 strains called by Kendall, Day and Walker *B. lactis-aerogenes* are clearly different in their behavior from the rest of the *B. mucosus-capsulatus* groups.

Average acidity (to neutral red) in glucose broth cultures of various colontyphoid organisms after various intervals

DAY	B. ALCAL-IGENES	DYSEN-TERY	TYPHOID	PARA-TYPHOID	MORGAN	B. COLI	B. LACTIS AERO-GENES	B. MUCO-SUS CAP-SULATUS	B. CLO-ACAE
1	- 0.4	2.1	3.0	3.0	2.8	3.8	3.7	2.2	2.0
3	- 0.8	2.7	3.3	3.8	3.8	4.4	4.2	1.6	1.6
6	- 1.0	2.7	3.5	3.8	3.7	4.8	4.8	1.3	0.9
9	- 1.1	2.7	3.6	3.7	3.9	5.0	4.6	1.0	- 0.1

The results, as tabulated above, indicate a progressive increase in acidity (a type III reaction) for dysentery, paratyphoid, Morgan, typhoid and colon bacilli, although it is interesting to note that Kendall, Day and Walker's figures indicate a distinct difference in the final end-point, the dysentery bacilli producing a lower, and the colon bacilli a higher, acidity than the typhoid, paratyphoid and Morgan groups. The forms called by Kendall, Day and Walker *B. lactis-aerogenes* appear by their fermentative activity to be of the *B. coli* type but those designated as *B. mucosus-capsulatus*, as well as the *B. cloacae* forms, show the characteristic type IV reaction of *B. aerogenes*.

Ayers and Rupp (1918) have recently brought forward direct and convincing evidence that the reversion of reaction exhibited in *B. aerogenes* cultures is due rather to the secondary decomposition of organic acids with the formation of basic carbonates than to neutralization by basic products of protein decomposition. They show that the reversion takes place even in a synthetic medium containing sodium ammonium phosphate as a source of nitrogen (in which case ammoniacal products could not possibly be sufficient to account for the phenomenon); and that it is accompanied by a rapid destruction of formic, acetic and other acids. Even with *B. coli* they noted as might be expected that acid formation does not run parallel with the destruction of glucose, formic acid remaining constant or being slightly reduced during the later stages of fermentation. The distinction between *B. coli* and *B. aerogenes* may therefore be considered as lying chiefly in the difference in rate between the preliminary decomposition of the sugars into acids and the secondary decomposition of the acids themselves; yet it is evidently a distinction that is clearly marked and of important systematic significance.

The slower and much less marked reversion which is observed if organisms giving the type III reaction are cultivated for a prolonged period may perhaps be due to a different process, an exhaustion of the fermentable carbohydrate and a subsequent decomposition of nitrogenous foodstuffs with the formation of alkaline substances. The same explanation may probably account for the reversion shown in milk cultures by the members of the paratyphoid group, the transient acidity produced being due to the fermentation of the small amount of glucose present.

It must of course be remembered that the course of such reactions as those described will vary within wide limits with variations in the composition of the medium employed. Clark and Lubs (1915) studied the effect of varying the concentration of carbohydrate and showed that with 0.5 per cent peptone and 0.5 per cent K_2HPO_4 and 0.1 per cent glucose *B. coli* shows a marked reversion, the curve of acidity by days resembling closely that given by *B. aerogenes* in the presence of 0.5 per cent glucose.

It has been shown by one of us (Kligler 1916) that the net result of the action of bacteria in a given medium not only depends on the relative amounts of peptone and glucose present but is influenced by the amount of phosphate as well.

CHARACTERISTICS OF COLON-TYPHOID BACTERIA OF GROUP I
(FERMENTING NO CARBOHYDRATES MORE COMPLEX THAN
THE HEXOSES)

The first well marked group of colon-typhoid organisms, as pointed out above, includes the forms of exceedingly low fermentative power which are capable of attacking only the simple hexoses if they ferment any carbohydrates at all, but are unable to produce acid in mannitol media.

The collection of cultures studied by us included 14 different strains belonging to this general group, which could be subdivided further by their action upon the hexoses and the formation of indol.

Five of the strains formed no acid in any carbohydrate media, the P_n value of glucose broth remaining over 7.0 and milk cultures turning gradually to a deeper and deeper blue.³ One strain (no. 439) showed a decolorization in milk after four weeks incubation, the color changing from deep blue to a pale chalky tint. These cultures were sent in to the Museum collection with the following names: *B. pneumoniae*, *B. dysenteriae*, *B. alcaligenes*, *B. ozenae*, *B. bronchisepticus*; but all of them have lost the power of attacking carbohydrates if they ever possessed it. All are indol negative⁴ and all fail to liquefy gelatin.⁵

³ Observations of the behavior of our cultures in milk were made under the following conditions. Certified milk was steamed for forty-five minutes in the Arnold sterilizer and left over night in the ice box for the cream to rise. The milk was then siphoned off, tinted with Kahlbaum's azolitmin, tubed and sterilized in the Arnold for twenty minutes on three successive days. After inoculation the cultures were incubated at 37°C. and observed after twenty-four and forty-eight hours and one, two, four and six weeks.

⁴ Indol production was studied by the following method which Marshall (1907) and others have shown to be an accurate and satisfactory one. A medium containing 0.3 gram tryptophane and 5 grams K_2HPO_4 in 1000 cc. of water was inoculated from a fresh twenty-four hour culture and incubated at 30° for forty-

It is of some interest to note that the names borne by three of these five strains would indicate that they did originally possess fermentative powers. Strain 27 of our collection was obtained from The Rockefeller Institute, as a Kral culture of *B. pneumoniae*; Strain 543 was isolated at the University of Pennsylvania, from a case of atrophic rhinitis identified as *B. ozenae* and successfully used as a vaccine; strain 177 was described as *B. dysenteriae* in the Journal of Experimental Medicine, 6, 181.

We have made no tests of motility on our strains, but Kendall, Day and Walker (1913) and Stewart (1917) describe *B. alcaligenes* as motile. Kühnemann (1911) claims that it has polar flagella, which if correct would remove it from the colon-typhoid group entirely. Petruschky's original description includes the statement that the flagella are peritrichic, and stresses the brownish growth upon potato. Berghaus (1905) who studied *B. alcaligenes* with some care bases the characterization of this species on the strongly alkaline reaction in milk, a brown coloration on potato, specific agglutination reactions, and the fact that it is an obligate aerobe. The species may be described as follows, and strain 439 of the American Museum collection (isolated from feces at the University of Pennsylvania) may be taken as a type.

B. alcaligenes Petruschky. Gram-negative non-spore-forming motile rods, producing thin translucent irregular colonies on

eight hours. One cubic centimeter of a 2 per cent alcoholic solution of p-dimethylamidobenzaldehyd was added drop by drop so as to mix with the medium, followed by a few drops of concentrated HCl, a reddish-purple color indicating the presence of indol. A comparison of the results obtained over a year before with the same cultures in a pepton medium showed that the results with the two procedures were identical.

⁵ Gelatin liquefaction was observed in small test tubes 1 cm. in diameter and 10 cm. long. Five cubic centimeters of standard gelatin made with Liebig's beef extract was placed in a tube and sterilized in the autoclave for five minutes under 20 pounds pressure. The tubes were inoculated by spreading a loopful of a twenty-four hour broth culture over the surface of the medium and they were incubated for twenty days at 20°C. in an atmosphere saturated with moisture. At the end of this time the depth of liquefied gelatin was estimated in centimeters.

gelatin and a brownish growth on potato. Reaction in carbohydrate media alkaline. Indol not produced. Gelatin not liquefied.

A second subdivision of the non-mannitol fermenting organisms, characterized by the decomposition of the hexoses with the production of acid but no gas, was represented in our collection by but two strains, the dysentery bacilli of Shiga and Kruse, respectively. They gave a type III reaction in glucose, mannose, fructose and galactose as indicated by the figures cited below.

Reaction (PH) produced by non-mannite-fermenting dysentery bacilli in glucose broth

	2 DAYS	4 DAYS	5 DAYS
Kruse.....	4.9	4.9	4.9
Shiga.....	5.0	5.1	4.9

No other carbohydrates were fermented; and in milk both strains produced a very faint initial reddening followed by a return to the original color of the medium. Indol was not formed, gelatin was not liquefied, and lead acetate media were not turned brown. Shiga originally described his dysentery organism as slowly motile but Kruse (1900), Vedder and Duval (1902) and all later observers agree that it is non-motile. All investigators who have studied the agglutination reactions of dysentery bacilli (among whom may be mentioned Pai and Krishnan, 1916 and Andrewes, 1918) report that the agglutinations of the Shiga type (including the Kruse and New Haven strains of non-mannite-fermenters) are sharply specific. Furthermore, as shown by Kruse, Flexner and Sweet (1906) and others the Shiga bacillus possesses the power, unique in the colon-typhoid group, of producing a soluble toxin. Nicolle, Debains and Loiseau (1916) note that this type produces in culture a characteristic odor of chestnut flowers. The Shiga type of dysentery appears to be an unusually definite and distinct one. Martin and Williams (1917) for example isolated 47 strains at

Cairo, all of which were true to type. While it is of course certain that both the mannit fermenting and non-mannit-fermenting types of dysentery bacilli are causally connected with the disease whose English name they bear, it is most desirable that they should be given definite Latin names in accord with scientific usage—particularly if, as we believe, they represent quite sharply differentiated bacterial types. It seems to us that the Flexner organism for instance is biologically more nearly related to *B. typhosus* than to the Shiga bacillus. Chester (1901) has given the Shiga type the name *B. shigae* and it may be characterized as follows.

B. shigae Chester. Gram-negative, non-spore-forming non-motile rods, producing thin translucent irregular colonies on gelatin. Form acid rapidly in media containing the hexoses, but not in other carbohydrates. Milk turned slightly acid and then neutral or slightly alkaline. Indol not produced. Gelatin not liquefied. Lead acetate media not browned. Produces a soluble toxin and exhibits characteristic agglutinative reactions. Found in human stools. The causative organism in one form of dysentery. Strain 197 of the American Museum collection is a type of this species. It was obtained by us from The Rockefeller Institute, labeled "Shiga, Japan, 293."

CHARACTERISTICS OF COLON-TYPHOID BACTERIA OF GROUP II (FERMENTING MANNITOL BUT NOT RHAMNOSE)

The second general group recognized above includes the forms which ferment mannitol and either xylose or arabinose but rarely both; and which produce acid but not gas in the sugars which are attacked. The arabinose-positive forms correspond to the Flexner group of dysentery bacilli and the arabinose-negative forms to *B. typhosus*.

Of the arabinose-fermenters we had only two strains in our series, both obtained from The Rockefeller Institute, strain 110 a Flexner-Harris strain, isolated by Flexner at Manila in 1900 and strain 196, a strain also isolated in the Philippines by Strong. Both were alike in forming acid in glucose, mannose, fructose,

galactose, mannitol and arabinose, but not in the other carbohydrates studied. The acid production was however rather of our type II than our type III order, being slightly more sluggish than in the case of most of the organisms studied, as indicated below.

Acidity (PH) produced by Flexner dysentery bacilli in glucose broth

	2 DAYS	4 DAYS	5 DAYS
Strain 110	6.0	5.4	5.5
Strain 196	5.6	5.4	5.6

Milk cultures were practically unchanged, although strain 110 showed a very slight reddening after two days, with reversion to the original neutral tint. Indol was produced and gelatin was not liquefied. Lead acetate media were not turned brown.

While the Shiga dysentery organisms constitute a clear and well-defined type the mannitol-fermenting strains are much more variable. Lentz reported that the Flexner strains fermented maltose and dextrin, while his Strong strains (all mannitol-fermenters) failed to do so. Hiss and Russell (1903) described their Y strain as failing to attack maltose; while the Flexner strain they studied acidified both maltose and sucrose. Park, Collins and Goodwin (1904) reported the same reactions for the Flexner type, while their Seal Harbor strain failed to attack either maltose or sucrose. Hiss (1904) recognized three subgroups of mannitol-fermenting dysentery bacilli as follows:

TYPES	MALTOSE	SUCROSE	DEXTRIN
Y (Hiss), Seal Harbor (Park), etc.....	—	—	—
Flexner, Strong	—	+	—
Flexner, Harris, Wolstein.....	+	+	+

On prolonged cultivation however the Y strains did attack maltose and sucrose (and a non-mannitol-fermenting Kruse strain attacked maltose!). Gay (1904) gave a similar classification. Morgan (1911) examined a series of about 50 dysentery-

like organisms, half from England and half from abroad and reported a considerable proportion of positive results (acid-production) in media containing sorbitol, arabinose and raffinose, in addition to the carbohydrates included in the table above. He concludes that

In the mannite-fermenting dysentery group (excluding the "Strong" strains) must be incorporated a large and probably ever-increasing number of strains reacting with striking uniformity to one test (i.e. agglutination with "Y" or Flexner serum) but differing from one another markedly when their fermentation properties and receptor mechanisms are minutely investigated.

Smith (1913) gives an elaborate classification of this group based on reactions in dulcitol, sucrose, sorbitol, dextrin and maltose. He calls the Y type dextrin positive, and in a later paper (Smith, 1915) gives the following reactions for the three common types.

	DULCITOL	MALTOSE	SORBITOL	DEXTRIN	ARABI- NOSE	RAFFINOSE
Y (Hiss).....	—	—	—	+	+	+
Strong.....	+	+	+	—	+	+
Flexner-Harris	—	+	—	+	+	+

It will be noted that these results differ in some respects from those cited above from Hiss and Gay, and it is probable that there were errors in the work of the earlier observers. Hort (1915) gives the same characteristics as those tabulated by Smith.

Recent observers have emphasized particularly the variability of the members of the mannitol-fermenting dysentery group and the difficulty of drawing sharp lines of distinction between them. Thus Pai and Krishnan (1916) report that Flexner and Y types cannot be clearly distinguished by their agglutination reactions. Martin and Williams (1917) studied 76 mannitol-fermenting dysentery bacilli which were constantly positive (acid-producing) in glucose, galactose and mannitol and constantly negative in lactose, dulcitol, inulin and adonitol, but which varied widely in their action on maltose, sucrose, dextrin,

raffinose, arabinose, rhamnose, sorbitol, glycerol and indol,—the same strain giving variable results at different times. Andrews (1918) and Thøtta (1919) have recently described types of dysentery-like organisms which indicate a confusing series of varieties within this group. Strain 110 of the American Museum collection, as originally a Flexner strain, should ferment maltose but as a matter of fact does not. Strain 196 as a Strong strain should ferment both maltose and sucrose but does not, giving us further evidence of the instability of fermentative powers of these organisms.

On the whole we are inclined to think that it is wiser not to burden the literature of this highly unstable group with a multiplicity of specific names based on fermentative irregularities, and believe that it would probably be best to include all the mannitol fermenters under the species *B. dysenteriae* with the principal sub-types as varieties. It may be noted that the agglutinative characters and pathogenic properties of these organisms are much more stable and appear to warrant the recognition of the group as a distinct entity.

The species and varieties may be defined as follows:

B. dysenteriae (Flexner). Gram negative, non-spore-forming rods. Non-motile. Producing characteristic translucent irregular colonies. Gelatin not liquefied. Indol generally produced. Acid production in media containing the hexoses and mannitol, and usually in arabinose. Lead acetate media not browned. Found in stools, causal agents of one form of dysentery.

Variety Hiss-Y. Dextrin fermented, maltose not fermented, sucrose not fermented.

Variety Flexner. Dextrin and maltose both fermented. Sucrose not fermented.

Variety Strong. Maltose and sucrose fermented but not dextrin.

The biological relationships of *B. dysenteriae* (Flexner) are very hard to surmise. It is related to *B. shigae* by lack of motility and by the nature of the disease which it produces; it is related to *B. typhosus* by the fermentation of maltose and mannitol; it is allied to both these organisms by failure to produce gas. On

the other hand the fact that it forms indol and sometimes attacks such complex carbohydrates as sucrose and raffinose would suggest a transition to *B. coli* and the lactose fermenters. Andrewes' (1918) three species of non-pathogenic *B. dysenteriae*-like bacilli may be connecting links along such a line.

Turning now to the organisms which ferment xylose but not arabinose, (*B. typhosus*) we find a much more compact and homogeneous group.

The typhoid bacillus, as pointed out in an earlier section, is characterized by active motility, failure to produce indol, and failure to ferment the more complex carbohydrates such as arabinose and rhamnose. Of our series of cultures, 24 belonged to this group. The fact that all but one of these strains came in to the American Museum collection under the name *B. typhosus* (the single exception being called *B. paratyphosus*) is good evidence of the fixity of this type. All of our 24 strains were alike in producing a rapid and complete acid reaction (type III) in the hexoses, and in mannitol, and xylose (in xylose the reaction reverted later slightly to about P_H 5.7), and in failing to attack lactose, sucrose, arabinose, rhamnose, dulcitol, or salicin. In litmus milk the medium was slightly reddened after two days but after two weeks turned neutral or slightly alkaline again. Indol was never formed, nor was gelatin liquefied. Lead acetate media were turned brown.

Sorbitol, which we did not study, is reported as acidified (Morgan, 1906, Smith, 1913, Robinson, 1915). So are maltose and dextrin (Smith, 1915, Harding and Ostenberg, 1912, Robinson, 1915). Smith (1915) states that raffinose is also attacked. This latter reaction has not been reported by other observers and seems improbable from the negative results in sucrose.

The typhoid bacillus is evidently a clearly marked and usually stable organism which may be defined as follows.

B. typhosus (Zopf). Gram-negative non-spore-forming rod. Actively motile. Forms translucent irregular colonies on gelatin media and faint nearly colorless growth on potato. Produces strong and prompt acid but no gas in media containing the hexoses maltose, mannitol, sorbitol, xylose and dextrin. Does

not attack arabinose, rhamnose or lactose. Produces a slight initial reddening of litmus milk, which after two weeks reverts to a neutral or slightly alkaline reaction. Fails to form indol or liquefy gelatin. Will not grow in asparagin-mannitol medium (Capaldi-Proskauer No. I). Does not reduce neutral red. Does cause browning of lead acetate media. Has low tolerance for acids but rather high tolerance for brilliant green dyes and alkaloids. Characteristic serum agglutination reactions. Found in human stools and urine as actual or potential cause of typhoid fever.

Our type of *B. typhosus* is strain 608, the Rawlings strain, obtained by Col. F. F. Russell from Colonel Leishman in 1908 and used in preparing the standard army vaccine.

There were two peculiar strains in our collection, both of which were sent in labeled *B. pyogenes-foetidus*. They were alike in giving the general fermentative reactions of *B. typhosus* but liquefied gelatin and casein rapidly.

CHARACTERISTICS OF COLON-TYPHOID BACILLI OF GROUP III
(FERMENTING MANNITOL, ARABINOSE AND RHAMNOSE,
GENERALLY FORMING GAS)

We may turn next to group III (see page 439) which includes the paratyphoid A types, fermenting the hexoses mannitol, maltose, rhamnose and arabinose (but not xylose) with the formation of gas, and producing like *B. typhosus* only a very slow reversion to an alkaline reaction in milk. Nine of the strains studied by us fell in this group. Six of them were sent in to the Museum collection labeled as Paratyphoid or specifically as Paratyphoid A; two as *B. pullorum*; and one as *B. alcaligenes*.

All fermented the hexoses, mannitol, rhamnose and arabinose. Two strains (nos. 294 and 322) attacked dulcitol. (Dulcitol is fermented by paratyphoid A according to Boycott, 1906, Morgan, 1906, Springer 1911, Smith 1915, and Jordan 1917). None of our strains produced acid in xylose, lactose, sucrose, salicin or inosite. Of the carbohyrates not studied by us, maltose is fermented by paratyphoid A according to Boycott 1906, Sac-

quépée and Chevrel 1906, Springer 1911, Smith 1915, Jordan 1917, and Krumwiede, Kohn and Valentine 1918; while dextrin is not attacked according to Jordan 1917, Weiss and Rice, 1917, Hulton-Frankel 1918 and Krumwiede, Kohn and Valentine 1918; and raffinose, inulin, erythritol and adonitol are not attacked according to Jordan 1917. Litmus milk was first turned slightly red but in the case of most of our strains by two weeks had reverted to a neutral or slightly alkaline reaction, very much as in the case of *B. typhosus*. Jordan (1917) states that his strains remained acid for two weeks, while Krumwiede, Pratt and Kohn (1916 b) report limits varying from five days to six weeks. Neutral red is said not to be reduced by Bainbridge 1919, but Sacquépée and Chevrel 1906, Hollande and Beauverie 1915, Nicolle, Raphael and Debains 1917 all report that this dye is decolorized. Lead acetate media were not browned by our cultures (as reported by Sacquépée and Chevrel 1906 and Hollande and Beauverie 1915). Gelatin was not liquefied and indol was not produced by any strain. Morgan (1906) stated that paratyphoid A produced indol but as shown by Marshall (1907) and Zipfel (1912) his methods of determining indol were unreliable. Among the minor characteristics of the paratyphoid A group may be mentioned the formation of agar colonies of a type intermediate between those characteristic of *B. typhosus* and *B. coli* respectively (Jordan 1917).

The designation of the paratyphoid bacilli as "A" and "B" has become firmly established in medical literature; but this terminology is quite clearly inadmissible from a systematic biological standpoint. The two forms are not varieties of one species but quite distinct forms, in spite of the fact that they produce a clinically similar disease. The A type is more nearly related to *B. pullorum* than to the B type; and the B type more nearly related to *B. enteritidis* and *B. suispestifer*, than to the A type of paratyphoid. It seems to us clear that definite specific names in a proper Latin form should be given to these organisms; and the name *B. paratyphosus* may properly be adopted for the A type to be defined as follows.

B. paratyphosus. Gram negative, non-spore-forming motile rods. Colonies on gelatin somewhat intermediate between the thin translucent irregular colonies of *B. typhosus* and the convex regular colonies of *B. coli*. Produces acid and gas in media containing the hexoses, mannitol, rhamnose, arabinose, maltose, sorbitol and sometimes dulcitol; but not in xylose, lactose, sucrose, salicin, inosite, dextrin, raffinose, inulin, or adonitol. Milk first turned slightly red and later (usually only after two weeks) neutral or slightly blue. Neutral red reduced, lead acetate media not browned. Gelatin not liquefied. Indol not produced. Characteristic serum agglutination reactions. Found in human stools and urine as actual or potential cause of one form of paratyphoid fever.

Culture 16 in our collection has been taken as a type of *B. paratyphosus*. It was obtained from the Rockefeller Institute in 1911 labelled Schottmüller A.

Clearly allied to *B. paratyphosus* is *B. pullorum*, described by Rettger in 1900 as the causative agent in bacillary white diarrhea of chicks. This organism, as shown by Rettger and Koser (1917), Hadley, Caldwell, Elkins and Lambert (1917) and Hadley, Elkins and Caldwell (1918) has all of the ordinary cultural and fermentative reactions of the A paratyphoids except that it is non-motile and fails to attack maltose or dulcitol, and that its agglutinative relations are closer with *B. typhosus* than with any of the paratyphoids. It may be defined as follows.

B. pullorum Rettger. Gram negative non-spore-forming non-motile rods. Colonies on gelatin somewhat intermediate between the thin translucent irregular colonies of *B. typhosus* and the convex regular colonies of *B. coli*. Produces acid and gas in media containing the hexoses, mannitol, rhamnose, arabinose and sorbitol; but not in maltose, dulcitol, xylose, lactose, sucrose, salicin, inosite, dextrin, raffinose, inulin or adonitol. Milk first turned slightly acid, later, but only slowly, neutral or slightly alkaline. Lead acetate not reduced. Gelatin not liquefied. Indol not produced. Exhibits group agglutination with *B. typhosus*. Causative agent of bacillary white diarrhea

in young chicks and found (without definite pathological symptoms) in ovaries of adult fowls.

Our strain 277 is a type of this species. It was isolated by Jones at Ithaca and sent to us by Parke Davis and Company in 1911 with the number 0233.

A special variety of this species has been described by Hadley (Hadley, Caldwell, Elkins and Lambert, 1917) as causing disease in adult fowls, differentiated from the typical *B. pullorum* by failure to form gas, but otherwise identical with it.

CHARACTERISTICS OF COLON-TYPHOID BACTERIA OF GROUP IV (FERMENTING MANNITOL, XYLOSE AND RHAMNOSE, GENERALLY PRODUCING GAS)

The fourth of the general groups into which we have divided the colon-typhoid series is that of which the B paratyphoids are the most typical examples. All of these forms ferment xylose and rhamnose and some of them arabinose as well, all produce a rather prompt alkalinity in milk, and all differ in agglutinative reactions from the A paratyphoid types. In general vigor of growth and fermentative power these forms stand nearer to *B. coli* than do any other of the non-lactose-fermenting organisms.

The first type of organism which we are inclined, with some doubt, to place in this group is the causative agent of fowl typhoid (*B. gallinarum*). This form, which was not represented in our own series, has the general fermentative reactions of the B. paratyphoids (see page 440) except that it attacks dextrin also, and that it fails to form gas. In view of the lack of correlation between gas production and power to attack various carbohydrates exhibited by the Morgan bacillus and the two varieties of *B. pullorum*, we are inclined to lay less stress on the former than on the latter characteristic. We cannot therefore agree with Hadley, Caldwell, Elkins and Lambert (1917) who believe that *B. gallinarum* is more closely allied to *B. typhosus* than is *B. pullorum*. All three organisms are however allied by their agglutinative reactions. According to Hadley, Elkins and Caldwell (1918) the milk reaction of *B. gallinarum* is of the B para-

typhoid type and the simultaneous fermentation of xylose and arabinose, a phenomenon which is so characteristic of paratyphoid B, seems to indicate that *B. gallinarum* may best be considered as a member of this group. It is certainly intermediate in its characteristics between *B. typhosus* and paratyphoid B; and it is of little consequence on which side of the arbitrary line between these types it may be placed.

The organism of fowl typhoid was described by Klein in 1889 as *B. gallinarum* and is probably identical with *B. sanguinarium* of Moore (1895). It may be characterized as follows.

B. gallinarum Klein. Gram-negative, non-spore-forming non-motile rods. Forming colonies on gelatin somewhat intermediate between the thin translucent irregular colonies of *B. typhosus* and the regular convex colonies of *B. coli*. Ferments the hexoses, mannitol, maltose, arabinose, xylose, dulcitol, dextrin, rhamnose, sorbitol, but not adonitol, salicin, lactose, sucrose or raffinose. Forms acid but no gas. Milk, first acid, quickly turning alkaline and developing the translucent appearance characteristic of paratyphoid B. Does not liquefy gelatin or produce indol. Exhibits group agglutination reaction with *B. typhosus*. Found as causative agent in fowl typhoid.

Hadley, Elkins and Caldwell (1918) describe two allied species, *B. psaffi* which differs from *B. gallinarum* in failing to ferment dextrin or dulcitol and in fermenting salicin; and *B. jeffersoni* which fails to ferment dulcitol and produces no change at all in milk.

B. avisepticus, the causative organism in fowl cholera, is a member of the Pasteurella group and does not belong in the colon typhoid series at all.

The more familiar organisms of the paratyphoid B group include, as pointed out above (p. 439) three distinct types, *B. suispestifer* (xylose + arabinose - inosite -), *B. enteritidis* (xylose + arabinose + inosite -), and paratyphoid B, itself (xylose + arabinose + inosite +).

Of the first of these types we had 5 strains in our collection, 3 of which were sent to us as *B. cholerae-suis*, one as *B. paratyphi*, and one as *B. sternbergii*. All fermented the hexoses, mannitol,

xylose and rhamnose but not arabinose, lactose, sucrose, salicin or inosite. Two attacked dulcitol and three did not. Milk was in all cases turned blue in two days and deepened in color progressively. Gelatin was not liquefied, indol was not produced, and lead acetate was not blackened. One strain of *B. cholerae-suis*, otherwise the same as the five mentioned, failed to ferment rhamnose.

Of the paratyphoid B forms which ferment arabinose as well as xylose and rhamnose, we had 24 strains in our collection. Five were originally sent in as paratyphoids, five as *B. enteritidis*, six as mouse and rat viruses of various sorts (*B. danysz*, *B. murium*, *B. murisepticus*), three as *B. abortivius*, and five under other names (*B. typhi-suis*, *B. typhosus*, *B. pullorum*, *B. icteroides*, *B. paracoli*). All were alike in fermenting the hexoses, mannitol, xylose, arabinose, and rhamnose and in failing to ferment lactose, salicin and sucrose. Dulcitol was attacked by nineteen strains and inosite by six (strains 22, 30, 169, 235, 237, 589). None formed indol or liquefied gelatin. Lead acetate was browned by all but the three strains of *B. abortivius*. Litmus milk was slightly reddened in two days but by the sixth day was always distinctly alkaline and became progressively more blue with a characteristic translucency.

These results obtained by us are in accord with the findings of previous students of this group, of whom the most important have been Boycott (1906), Bainbridge (1909), Harding and Ostenberg (1912), Jordan (1917), Krumwiede, Pratt and Kohn (1917), and Krumwiede, Kohn and Valentine (1918).

All observers agree that the paratyphoid organisms are motile. Boycott (1906), Smith (1915) and Jordan (1917) point out that they ferment maltose, and sorbitol; Jordan (1917) and Krumwiede, Kohn and Valentine (1918) report failure to attack raffinose, adonitol, dextrin, inulin and erythritol. The reduction of neutral red is described by Sacquépée and Chevrel (1906), Bainbridge (1909), and Hollande and Beauverie (1915). High resistance to green dye is reported by various observers.

Of the three species included in this group the first, distinguished by failure to attack arabinose and generally negative

results in dulcitol, is the organism isolated by Salmon and Smith from cases of hog cholera in 1885. This form has been confused with an organism of the hemorrhagic septicemia group associated with swine plague from which it is quite distinct. The type found in hog cholera should bear the name *B. suipestifer* and the swine plague organism that of *B. suissepticus*. *B. suipestifer* according to Jordan (1917) is a form quite constantly of porcine origin, while both *B. enteritidis* and paratyphoid B are commonly isolated from human sources. It is distinguished from the human forms by failure to brown lead acetate media and, according to Krumwiede, Kohn and Valentine (1918) by the power to reduce Andrade's indicator in glucose-serum-water. It may be defined as follows, culture 258 obtained from Parke Davis and Company in 1911 labelled "Boxmeyer, Belle Plain, 1903 No. 053" being taken as the type.

B. suipestifer Kruse. Gram negative, non-spore-forming, motile rods. Forming colonies on gelatin intermediate between the thin translucent irregular colonies of *B. typhosus* and the regular convex colonies of *B. coli*. Ferments the hexoses, mannitol, maltose, xylose, rhamnose and sorbitol with formation of gas. Does not ferment arabinose, lactose, sucrose, salicin, inosite, raffinose, adonitol, dextrin, inulin or erythritol. Generally fails to attack dulcitol. Turns milk first slightly acid, reverting in five days to an alkaline reaction, the color of the medium deepening with the development of a translucent appearance. Neutral red and malachite green reduced, lead acetate not blackened. Indol not produced. Gelatin not liquefied. Group agglutination with paratyphoid B. Found in intestines of hogs and as secondary invader of tissues in hog cholera.

B. enteritidis is distinguished from *B. suipestifer* by fermentation of arabinose, general fermentation of dulcitol, and blackening of lead acetate media, and from paratyphoid B by failure to ferment inosite. It exhibits distinct and characteristic serum agglutinative reactions. It may be defined as follows.

B. enteritidis Gaertner. Resembles *B. suipestifer* in all cultural characters except that arabinose is always, and dulcitol

generally fermented, and that lead acetate media are blackened. Exhibits characteristic serum agglutination reactions which distinguish it from either *B. suipestifer* or paratyphoid B. Found in human intestine and as causative factor in outbreaks of food poisoning. The type is our strain 18, a Gaertner strain obtained from the Rockefeller Institute in 1911.

Finally there remains for consideration the true paratyphoid B organism which is distinguished from *B. enteritidis* by the fermentation of inositol and by its serological reactions. So far as we are aware no one has ever given this very distinct type a specific name in proper Latin form; and since the identification of the paratyphoids merely as "A" and "B" is quite misleading as to their true biological relationships, we suggest the name *B. schottmulleri* for the paratyphoid B organism, to be defined as follows.

B. schottmulleri. Resembles *B. suipestifer* in all cultural characters except that arabinose and inositol are always fermented and dulcitol is generally fermented and that lead acetate media are browned. Its agglutinative reactions distinguish it from *B. enteritidis*. Found in human intestines and urine and as causative agent in paratyphoid fever and food poisoning outbreaks. The type is our strain 22, a Schottmüller strain obtained from the Rockefeller Institute in 1911.

The various forms of mouse and rat virus appear not to be distinct entities according to Bainbridge (1909), Savage (1912) and Krumwiede, Pratt and Kohn (1917); and our own results bear out their view that some of these viruses belong to each of the three species listed above, the Danyz virus being a variety of *B. enteritidis*.

The peculiar organism known as the Morgan bacillus may perhaps best be considered here, although its exact relationships are obscure, since it possesses the power of forming gas but can attack only a limited number of carbohydrates. Five of our cultures were of this type. Two of these strains came to the Museum as Morgan bacilli while the others were labeled respectively *B. communis*, *B. cuniculicida* and *B. pseudotuberculosis*, but all were alike in their failure to ferment the higher carbohydrates and in the production of a small but definite amount of

gas (about 5 per cent) in glucose, mannose, fructose and galactose. The acid production in the hexoses was vigorous in all these five strains when they were first tested in 1917, but when re-examined in 1919 one of them (strain 586) had lost its power to attack any of the sugars. This observation, together with the fact that one of the strains (strain 139) which attacked the hexoses, but only the hexoses, in both our tests was originally obtained from D. D. Jackson as *B. communis* A (fermenting mannitol, dulcitol, lactose and raffinose) again suggests a loss of fermentative power during prolonged cultivation in the laboratory. In milk cultures all of the strains produced an immediate alkaline reaction, later decolorizing the litmus to some extent so as to produce a chalky blue appearance. This fact is probably associated with the rapid decomposition of protein characteristic of this form. Kendall, Day and Walker (1913) note a production of 4.2 mg. of NH_3 per 100 cc. of glucose broth for the Morgan bacillus in one day, as against less than 0.1 mgm. for *B. typhosus* and 0.2 for the paratyphoids. All of our strains formed indol and failed to liquefy gelatin.

Morgan (1906) describes this organism as motile and differentiates it from the hog cholera type by its reaction in litmus milk, active production of indol and failure to ferment maltose, arabinose and dextrin. Organisms of this type have been studied by Lewis (1912), Alexander (1912) and particularly by Graham-Smith (1912). The latter investigator prepared an elaborate system of classification of the non-lactose-fermenting bacteria, his type G including the forms which produce gas in glucose but not in mannitol media, being the most common of all the types in the lactose-negative group. Tribondeau and Fichet (1916), who isolated the Morgan bacillus from 13 cases of dysentery originating in the Dardanelles note, in addition to the characters described above, that it produces a fluorescence in neutral red broth and rapidly blackens lead acetate. They found several strains of related organisms which at first fermented maltose and sucrose but which later lost this power.

It is evident that the Morgan bacillus, wherever its closest relationships may lie, constitutes a fairly definite type, of com-

mon occurrence in the human intestinal canal. So far as we are aware it has nowhere been given a scientific name in proper form; and we have therefore called it *B. morgani*, characterizing the species as below and considering culture 692 of the American Museum collection as its type. This strain was isolated from the stool of an infant at the Providence City Hospital by H. E. Smiley.

B. morgani. Gram-negative, non-spore-forming motile rods, producing thin translucent irregular colonies on gelatin. Rapid formation of acid and slight gas production in media containing the hexoses. Milk turned gradually blue. Indol formation vigorous. Gelatin not liquefied. Produces fluorescence in neutral red broth and blackening in lead acetate media. Found in normal and diarrheal stools.

This organism would seem to be allied to *B. shigae* by its limited fermentative powers, to *B. dysenteriae* (or *B. coli*) by indol production, and to the paratyphoids by the formation of gas. It may perhaps represent an extreme variant of the variable *B. dysenteriae* group but we have considered it with the paratyphoids on account of its gas production.

One other type of non-lactose-fermenting organism often considered as a member of the colon-typhoid group is the *B. proteus*; but on account of the fundamental differences in morphology and metabolism (fermentation of sucrose but not lactose and very vigorous decomposition of proteins) we believe the *Proteus* forms should not form a part of this series at all.

CHARACTERISTICS OF COLON-TYPHOID BACTERIA OF GROUP V (FERMENTING LACTOSE AND THE SIMPLER CARBOHYDRATES WITH PRODUCTION OF EQUAL VOLUMES OF CO₂ AND H₂)

This group of organisms, of which *B. coli* is the most familiar example, is distinguished from all the organisms of the dysentery-typhoid-paratyphoid series by the power of fermenting lactose and from the *B. aerogenes* types by the fact that in its fermentation it produces equal volumes of CO₂ and H₂ (low ratio fermentation of Clark and Lubs). Its members differ from the

typhoid and paratyphoid organisms in less active motility, in more vigorous growth on media (with more convex and regular colonies), in strong reducing action, in the formation of indol, and in characteristic differences in resistance to various antiseptics.

In the Museum collection we found 42 different strains belonging to this general group. All of them fermented the hexoses, mannitol, xylose, arabinose, rhamnose, and lactose, with the production of rapid and permanent acidity and gas. None attacked inositol while with salicin, dulcitol and sucrose results were variable. All acidified and coagulated milk promptly, a firm clot being generally formed in six days with marked decolorization of the litmus. Indol was formed by all but 10 strains. Gelatin was not liquefied.

In regard to other reactions which we did not study it may be noted that maltose is fermented, according to Levine and other workers; that adonitol is generally not attacked (Mac Conkey, 1909, Rogers, Clark and Lubs 1918); and that starch, glycogen, inulin and dextrin give negative results according to Chantemesse and Widai (1891), Drigalski and Conradi (1902), Burk (1907), MacConkey (1909), Rogers, Clark and Evans (1914, 1915), and Levine (1916). Murray (1916) however reports that a fair proportion of strains from bovine feces do attack inulin.

Twort (1907) in a study of the utilization of a large series of unusual glucosides found that *B. coli* generally attacked euonymin, iridin, senegin, coniferin, arbutin, salicin, syringin, quillajinic acid, populin, camellin, and globularin. The hydrolytic splitting of esculin with the production of sugar and a substance called esculetin which reacts with iron citrate to produce a brown color has been used by Harrison and van der Leek (1909) and others as a test for this group. The colon bacillus generally reduces nitrates and neutral red (Rothberger, 1898); but does not brown lead acetate media (Sacquépée and Chevrel, 1905, Burnet and Weissenbach, 1915). It produces an acid reaction in Capaldi and Proskauer's medium 1 but not in medium 2. It has a relatively high resistance to acid (Hankin, 1899, Winslow and Lochredge, 1902) potassium tellurite (Davis, 1914), and cholesterol (Manfredi, 1917); but a relatively low resistance to

the green dyes (Loeffler, 1903, 1906, Lentz and Tietz, 1903, 1905, Krumwiede and Pratt, 1914), caffeine (Hoffman and Ficher, 1904), and bile salts (Jackson and Melia, 1909).

The further subdivision of the colon group of bacteria was first attempted on the basis of the fermentation of sucrose. Germano and Maurea (1893) for example distinguished one type which fermented sucrose and decolorized jequirity solution and another which failed to give either of these reactions. Smith (1893, 1895a) in his classic investigations emphasized the importance of the sucrose-positive and sucrose-negative varieties. Durham (1901) gave the sucrose-positive form the name *B. communior*. Winslow and Walker (1907) and Howe (1912) pointed out that the forms which attack sucrose also attack raffinose and vice versa.

Very elaborate classifications of the colon group were developed by MacConkey (1905), Bergey and Deehan (1908), and Jackson (1911), based primarily on fermentation of sucrose and dulcitol and secondarily on fermentation of adonitol and inulin, the Voges-Proskauer reaction, motility, indol formation and liquefaction of gelatin. The principal types recognized by these authors were as follows.

- I. Sucrose - Dulcitol - *B. acidi-lactici*
- II. Sucrose - Dulcitol + *B. coli-communis*
- III. Sucrose + Dulcitol - *B. coscoroba* [*B. aerogenes* (V. - P. +), and *B. cloacae* (V. - P. +, Gelatin +) also belong here]
- IV. Sucrose + Dulcitol + *B. communior* [*B. neapolitanus* (non-motile) and *B. pneumoniae* also belong here]

These classifications were all defective in grouping the Voges-Proskauer positive, indol negative, gelatin positive organisms with the various fermentative types of the true colon group. We now know that these forms belong to the high ratio (*B. aerogenes*) group and should not be placed with *B. coli* and *B. communior*. *B. acidi-lactici*, *B. coli*, *B. coscoroba* and *B. communior* may however be considered to represent distinct types, if the fermentation of dulcitol is really a characteristic of specific importance. There seems grave doubt however whether this

alcohol should be given a prominent place in classification; for as pointed out above dulcitol throughout the colon-typhoid series shows less correlation with other properties than any other carbohydrate and under experimental conditions shows the most marked tendency to spontaneous variations.

Rogers, Clark and Evans (1914), in a study of colon organisms from bovine feces, found that sucrose and raffinose fermentations are directly and almost perfectly correlated, and that the sucrose positive forms are generally dulcitol positive and adonitol negative, while the sucrose negative forms vary in both dulcitol and adonitol. The sucrose negative forms may be divided (although the authors do not point this out) into a dulcitol + adonitol - group and a dulcitol - adonitol + group. Kligler (1914) emphasized the value of salicin as a differential test substance, finding dulcitol inversely correlated with salicin among the sucrose positive forms and positively correlated among the sucrose negative forms. Failure to attack glycerol was most common among the salicin-fermenters.

The most extensive study of this group of organisms is that conducted by Levine (1917). This investigator worked with 333 strains isolated from soil, sewage and the feces of men, horses, sheep, pigs and cows and devoted special attention to the mutual correlation of the various characters studied. One hundred and eighty-two of the strains were of the methyl-red-positive, Voges-Proskauer-negative type. Sucrose and raffinose were almost perfectly correlated and the author divides his methyl red positive organisms first on the sucrose fermentation. The sucrose negative forms are next subdivided according to their action on salicin, giving *B. coli* (sucrose - salicin +) and *B. acidi-lactici* (sucrose - salicin -) as species. Among the sucrose positive strains the most natural division, according to correlated characters, is between a motile type *B. communior* and a non-motile series which can be further subdivided into a salicin positive species *B. neapolitanus* and a salicin negative species, *B. coscoroba*. *B. communior*, *B. neapolitanus* and *B. coscoroba* were much more common in animal than in human feces, while *B. coli* and *B. acidi-lactici* were commonly isolated from human sources.

It is impossible to make a direct and comprehensive comparison of the results of these various investigators since each one used some tests not applied by the others. In our own investigations we did not study motility or the fermentation of adonitol and salicin. The following characteristics would appear to be indicated for the principal types recognized by Kligler and Levine.

	SUCROSE	SALICIN	DULCITOL	ADONITOL	MOTILITY
<i>B. neapolitanus</i>	+	+	-	-	-
<i>B. communior</i>	+	-	+	-	+
<i>B. coscoroba</i>	+	-	+	-	-
<i>B. coli-communis</i>	-	+	+	-	+
<i>B. immobilis</i>	-	+	+	-	-
<i>B. Grünthal</i>	-	-	-	+	+
<i>B. acidi-lactici</i>	-	-	-	+	-

All recent observers are agreed as to the superior value of salicin as compared with dulcitol for the primary subdivision of this group; and Levine's main classification, based on so large a series of strains can safely be accepted as in the main a correct one. We cannot feel certain however that the presence or absence of motility is a sufficient basis for the establishment of species, in view of the highly inconstant results obtained by other students of this property. We are therefore inclined to recognize four distinct species, as characterized below, with three varieties, based on motility.

B. neapolitanus Fraenkel. Gram-negative non-spore-forming rod. Non-motile. Vigorous growth on media, colonies more regular and convex than those of *B. typhosus*. Produces prompt and permanent acidity and gas composed of equal volumes of CO₂ and H₂ in media containing the following substances; the hexoses, maltose, mannitol, xylose, arabinose rhamnose, lactose, sucrose, salicin and esculin, but not as a rule dulcitol or adonitol, and never inosite, starch, glycogen, inulin or dextrin. Turns milk strongly acid and coagulates it in six days at 37°. Generally forms indol but does not liquefy gelatin. Produces an acid reaction in Capaldi-Proskauer medium 1 but

not in medium 2. Does not give Voges-Proskauer reaction. Reduces neutral red and generally nitrates. Does not brown lead acetate media. Exhibits high tolerance to acids, potassium tellurite and cholesterin, but relatively low tolerance to the green dyes, alkaloids and bile salts. Found in feces of higher animals and man, particularly the former.

Our type of this species is strain 126, isolated from a urinary fistula in 1911 and received by us from Dr. Jackson of the Mt. Prospect laboratory as *B. aerogenes* A2.

B. communior Durham. Differs from *B. neapolitanus* in failing to ferment salicin, in generally fermenting dulcitol and in being sluggishly motile. Our type is 137 isolated from feces by Dr. Frazer in 1911 and sent to us by Jackson as *B. communior*.

var. *coscoroba*, differs from *B. communior* in lacking motility.

B. coli Escherich. Differs from *B. neapolitanus* as described above in failing to ferment sucrose and raffinose, in generally fermenting dulcitol, and in exhibiting sluggish motility. Specially abundant in human feces. Type, 125 isolated from a case of cystitis in 1910 and sent to us by Jackson as *B. communis* *B*.

var. *immobilis* differs from *B. coli* in being non-motile.

B. acidi-lactici Grotenfelt. Differs from *B. neapolitanus* in failing to ferment sucrose, raffinose or salicin and in generally fermenting adonitol. Specially abundant in human feces. Type, 131 from Jackson as *B. acidi-lactici* *B*. Isolated from feces.

var. *Grünthal*. Differs from *B. acidi-lactici* in being motile.

All of the type specimens listed above are described by Jackson (1911). He states that type 137 does not ferment mannitol but we find that it does.

The organisms in our collection did not show by any means a clear cut correlation between the fermentation of sucrose, salicin and dulcitol. Classifying them according to their action upon sucrose and salicin we found 15 strains of *B. neapolitanus* (of which only 5 were negative in dulcitol), 4 of *B. communior* (of which 3 were negative in dulcitol), 16 of *B. coli* (of which 10 were positive in dulcitol), and 7 of *B. acidi-lactici* (of which 4 were negative in dulcitol). The names under which these organisms were sent to us corresponded fairly well with the species as

identified except that the *B. neapolitanus* series included two strains called *B. aerogenes*, one *B. pneumoniae*, and one *B. ozenae*, one *B. sternbergii* and one *B. bovisepcticus*; the *B. communior* series one *B. bronchicanis*; the *B. coli* series, a *B. diphtheriae-columbarum*, two *B. cholerae*, one *B. astheniae*, one *B. anaerogenes*, one *B. aerogenes*, and 3 *B. acidi-lactici*; and the *B. acidi-lactici* series two *B. coli*, one *B. voldagsen*, one *B. danysz*, and one *B. dysenteriae* (!).

CHARACTERISTICS OF COLON TYPHOID BACTERIA OF GROUP VI
(FERMENTING LACTOSE AND THE SIMPLER CARBOHYDRATES
WITH PRODUCTION OF TWO OR MORE VOLUMES OF
CO₂ TO ONE OF H₂)

The last group of the colon-typhoid series, of which *B. aerogenes* and *B. cloacae* are the principal types, was recognized by Escherich (1885) on the basis of greater plumpness of the cell form, lack of motility and more rapid coagulation of milk, by Smith (1893a) on the basis of heavier growth and tendency to capsulation, and later (Smith 1895a) on rapid gas production and higher ratio of CO₂ to H₂. Durham (1901) notes the fermentation of starch and inulin and the Voges-Proskauer reaction as characteristic, Grimbert and Le Gros (1900) and Jordan (1903) the failure to form indol. Finally Harden and Walpole (1905) and Rogers, Clark and Davis (1914) demonstrated conclusively the fundamental difference in carbohydrate metabolism between these forms and those of the *B. coli* type. Rettger (1903) showed that the differences in protein metabolism are equally distinct, *B. aerogenes* being much less active in this respect forming mercaptans, skatol, phenols, aromatic oxy-acids and skatol-carbolic acid much more slowly. Ferreira, Horta and Paredes (1908) report that *B. aerogenes* and *B. cloacae* ferment sucrose but not dulcitol and give a rose color with no luster on the Endo medium. Rivas (1908) points out that *B. aerogenes* exhausts the carbohydrate constituent in glucose broth much more rapidly than does *B. coli*. Rogers, Clark and Davis (1914) described a series of high gas ratio cultures isolated

from milk, and pointed out that these forms attack sucrose, raffinose, and starch and liquefy gelatin more frequently than do the *B. coli* forms, and on the other hand attack dulcitol and glycerol less often. Rogers, Clark and Evans (1915) studied 166 high ratio cultures from grains and described six different types of which the most abundant had the following characteristics.

Gelatin-liquefying forms. Not capsulated, indol negative, sucrose positive, raffinose positive, mannitol positive, dulcitol positive, glycerol generally positive, starch negative, inulin negative, adonitol negative.

Gelatin-non-liquefying forms. Sometimes capsulated, sometimes fermenting starch, inulin and adonitol, rarely fermenting mannitol or dulcitol, otherwise as above.

Rogers, Clark and Lubs (1918) describe a series of high ratio strains from human feces as generally indol negative, sucrose, raffinose, mannitol and adonitol positive, inulin negative, and generally dulcitol negative. According to these results the high ratio type common in human feces differs from that normal on grains by the fermentation of mannitol and adonitol.

Levine (1916b) states that *B. aerogenes* usually ferments sucrose, raffinose, salicin, glycerol, dextrin and starch, but rarely dulcitol. In a later paper (Levine 1917) he confirms the conclusion of Kligler (1914) (opposed to that of Rogers, Clark and Evans) that gelatin liquefaction and glycerol fermentation are negatively correlated. Of 151 strains of the high ratio type (isolated from soil or in a few cases from sewage) all fermented mannitol, and almost all sucrose, raffinose, and salicin. He recognizes two species, *B. aerogenes*, which rarely liquefies gelatin, is non-motile and forms gas from glycerol and starch; and *B. cloacae* which is a liquefying organism failing to ferment either glycerol or starch.

In general it is evident that the organisms of this group may be subdivided into a type which liquefies gelatin and one which fails to do so. The former, *B. cloacae*, is not capsulated, and does not ferment starch, inulin or adonitol, while *B. aerogenes* is often capsulated and sometimes ferments starch and inulin

and adonitol. According to Rogers, Clark and Lubs the grain type of *B. aerogenes* differs from the fecal type in its failure to attack mannitol and dulcitol. Twort (1907) reports the frequent fermentation by *B. aerogenes* of a number of glucosides not attacked by *B. coli* such as cerberid, periplocin, cathartinic acid, amygdalin, sapotoxin, saponin, bryanin, convallamarin, digitalin, strophanthin, coronillin, gratiolin and phloridzin.

Our own series of cultures included 8 of the *B. cloacae* and 23 of the *B. aerogenes* type. All gave positive results in the hexoses, mannitol, xylose, arabinose, rhamnose, lactose, salicin and sucrose. All coagulated and decolorized milk, the clot being formed by 19 strains in six days at 37°, by the other 12 more slowly. Only 6 strains formed indol. All but 1 were alkaline to methyl red and all but 8 gave the Voges-Proskauer reaction. Only 9 fermented dulcitol and 16 fermented inositol. A special study showed that our gelatin-liquefying strains all failed to ferment glycerol, while the *B. aerogenes* did attack this substance.

We may therefore recognize at least two species in this group, as follows.

B. cloacae Jordan. Gram-negative non-spore-forming rod. Non-motile. Vigorous growth on media, colonies even more regular and convex than those of *B. coli*. Ferments the hexoses, maltose, mannitol, xylose, arabinose, lactose, rhamnose, sucrose, raffinose, and salicin, often inositol, but generally not dulcitol, glycerol, starch, inulin or adonitol. Produces vigorous and complete destruction of the carbohydrates which it attacks forming acetyl-methyl-carbinol (Voges-Proskauer reaction) and at least twice as much CO₂ as H₂, the reaction of the medium becoming moderately acid and then reverting to a lower degree of acidity. Acidifies and coagulates milk but not quite so promptly as *B. coli*. Fails to form indol. Liquefies gelatin slowly. Found in human and animal feces, sewage and soil. Our type of this species is type 23 isolated by Jordan from the Chicago Drainage canal in 1899.

B. aerogenes Escherich. Gram-negative non-spore-forming rod. Generally non-motile. Frequently capsulated. Growth on media vigorous, colonies convex and often viscid in texture.

Ferments the hexoses, maltose, xylose, arabinose, rhamnose, lactose, sucrose, raffinose, salicin, usually glycerol, starch, and inulin, and sometimes inositol and adonitol, dulcitol and mannitol. Produces vigorous and complete destruction of the carbohydrates, forming acetyl-methyl-carbinol (Voges-Proskauer reaction), and at least twice as much CO_2 as H_2 , the reaction of the medium becoming moderately acid and then reverting to a lower degree of acidity. Acidifies and coagulates milk but not quite so promptly as *B. coli*. Fails to form indol or liquefy gelatin. Found in human and animal feces and sewage but particularly in soil and on grains.

Our type strain is type 240 received from Johns Hopkins in 1911 with following history—"probably a descendant of the original capsule bacillus of Pfeiffer . . . reaction identical with *B. aerogenes*."

A variety which fails to ferment mannitol and adonitol is the commonest form on grains.

B. aerogenes represents the extreme of fermentative power in the colon-typhoid series; and on the assumption that the course of evolution has been marked by progressive loss of fermentative power and acquisition of the parasitic habit it may be considered the most primitive type of the whole group. It has been suggested by one of us (Kligler, 1917) that three different lines of evolution may have started from this type, the first leading through capsulated streptococci to the diverse forms of streptococci and pneumococci of the present day, the second through *B. coli*, *B. typhosus* and the dysentery organisms to the hemorrhagic septicemia group, and the third through *B. cloacae* and *B. proteus* to the saprophytic spore-bearing and pigment bacteria.

There is one group of organisms usually considered to be related to *B. aerogenes* whose affiliations appear to be obscure. These are the capsulated forms frequently met with in association with certain pathological conditions. Capsulated bacilli were first described by Friedlander from pneumonia in 1883; and important studies of this group have been made by Fricke (1896), Strong (1899), Perkins (1904) and Coulter (1917).

All of these workers assumed that the pathogenic capsulated bacilli with which they worked (*B. pneumoniae*, *B. ozenae*, *B. rhinoscleromatis*) were related to *B. aerogenes*; but all agree that they differ from *B. aerogenes* in producing a more distinct capsule and colonies of a translucent syrupy consistency; in forming indol; and in fermenting sucrose but not lactose. The last property is a very rare one in the colon-typhoid group, although the fermentation of sucrose but not lactose is characteristic of the *Proteus* bacilli. In our Museum collection we had a number of organisms which were sent to us bearing the names of the capsulated pathogenic forms; but none of them exhibited the characteristics described by Perkins, Strong and Coulter. Of three cultures sent to us as *B. pneumoniae* one was finally classed as *B. alcaligenes*, one as *B. neapolitanus*, and one as *B. aerogenes*. Of two sent in as *B. ozenae*, one was *B. alcaligenes* and one *B. neapolitanus*. Two sent in as *B. capsulatus* and one as *B. rhinoscleromatis* all proved to be *B. aerogenes*. Thus of eight strains originally believed to be members of this group, four were of the methyl red negative, Voges-Proskauer positive type, while two belonged at the other extreme of the paratyphoid series, with no fermentative powers at all. It seems evident, either that we are dealing with an extraordinarily variable group or that forms which are not really related have been identified as of this type merely because of the possession of a capsule. (Fletcher, 1918, has recently described capsulated forms of paratyphoid and dysentery bacilli). The evidence brought forward by Strong (1899), Perkins (1904) and Coulter (1917) is so completely concordant as to make it clear that there is a distinct type of capsulated bacillus which ferments sucrose and not lactose and possesses considerable pathogenic powers; but in view of the incompleteness of our knowledge of its other reactions we are somewhat uncertain of its relations to the colon-typhoid group.

REVIEW OF THE GENERAL CHARACTERISTICS OF THE SPECIES OF THE COLON-TYPHOID GROUP

The principal characters of the species of colon-typhoid bacteria as defined above are presented for convenient comparison in the table below, and the fermentative relationships of certain of the more important forms are presented in figure I.

If *B. aerogenes* be taken as a representative of the most primitive type it is evident that there is a more or less steady decrease

SPECIES	HEXOSE	MALTOSE	MANNITOL	XYLOSE	ARABINOSE	RHAMNOSE	SORBITOL	DULCITOL	LACTOSE	SALICIN	SUCROSE	RAFFINOSE	INOSITE	DEXTRIN	GAS	VOGES-PROSKAUER	METHYL RED	MILK	GELATIN	INDOL	LEAD ACETATE	MOTILITY	PATHOGENIC
<i>B. aerogenes</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Coag.	-	-	-	-	-
<i>B. cloacae</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Coag.	+	-	-	-	-
<i>B. neapolitanus</i> ..	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Coag.	-	+	-	-	-
<i>B. communior</i> ..	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Coag.	-	+	-	-	-
<i>B. coli</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Coag.	-	-	-	-	-
<i>B. acidilactici</i> ..	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Coag.	-	+	-	-	-
<i>B. morgani</i>	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	al.	-	+	+	+	+
<i>B. schottmulleri</i> ..	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	ac. al.	-	-	+	+	+
<i>B. enteritidis</i>	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	ac. al.	-	-	+	+	+
<i>B. suipestifer</i>	+	+	+	+	-	+	+	+	-	-	-	-	-	-	+	+	+	ac. al.	-	-	-	+	+
<i>B. gallinarum</i> ..	+	+	+	+	+	+	+	+	-	-	-	-	-	+	-	+	+	ac. al.	-	-	-	-	+
<i>B. pullorum</i>	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	ac. al.	-	-	-	-	+
<i>B. paratyphosus</i> ..	+	+	+	-	+	+	+	+	-	-	-	-	-	-	+	+	+	ac. al.	-	-	-	+	+
<i>B. typhosus</i>	+	+	+	+	-	-	+	+	-	-	-	-	-	+	-	+	+	ac. al.	-	-	+	+	+
<i>B. dysenteriae</i> ..	+	+	-	+	+	+	+	+	-	-	+	+	+	+	-	+	+	ac. al.	-	+	-	-	+
<i>B. shigae</i>	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	ac. al.	-	-	-	-	+
<i>B. alcaligenes</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	al.	-	-	+	+	-

of fermentative power, down through the colon, paratyphoid and typhoid groups to *B. shigae* and *B. alcaligenes*. It cannot however be maintained that this progression necessarily or even probably represents the exact line of evolutionary development. In certain respects (as in its action on lead acetate media) *B. typhosus* seems more closely allied to *B. schottmulleri* than to *B. paratyphosus*. *B. dysenteriae* as noted exhibits highly variable reactions on maltose, xylose, arabinose, dextrin, rhamnose,

salicin and sucrose, which have therefore been left out of the chart entirely. *B. morgani* is another highly variable and unstable type, as is also *B. pullorum*; while the capsulated pathogenic forms allied to the Friedlander bacillus form an exceedingly puzzling complex. On the other hand *B. shigae* and *B. typhosus*

	<i>B. aerogenes</i>	<i>B. cloacae</i>	<i>B. neapolitana</i>	<i>B. commanior</i>	<i>B. coli</i>	<i>B. acid-lactici</i>	<i>B. schottmulleri</i>	<i>B. enteritidis</i>	<i>B. suispestifer</i>	<i>B. paratyphosus</i>	<i>B. typhosus</i>	<i>B. dysenteriae</i>	<i>B. shigae</i>	<i>B. alcaligenes</i>
Hexoses														
Mannitol														
Maltose														
Corbitol														
Gas Production														
Rhamnose														
Arabinose														
Xylose														
Lactose														
Salicin														
Sucrose														
Raffinose														
Voges-Proskauer Reaction														

FIG. I

are species of very definite and constant characteristics; while *B. schottmulleri*, *B. enteritidis*, *B. suispestifer*, and *B. paratyphosus*, as well as the chief colon-aerogenes types, can be identified with reasonable ease.

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A CONTRIBUTION TO THE BACTERIOLOGY OF A FUSO-SPIRILLARY ORGANISM, WITH SPECIAL REFERENCE TO ITS LIFE HISTORY¹

RALPH R. MELLON

Department of Pathology, Hahnemann Hospital, Rochester, New York

Received for publication, March 7, 1919

This study has as its origin an obscure clinical case of generalized infection with a fuso-spirillary organism. In this communication I do not deem it desirable to allocate its place in the scale of organic life with finality, partly because of present classificatory readjustments that are being carried out by the Society of American Bacteriologists, and partly because of additional work that we are prosecuting with these and related organisms. The term fusospirillary I shall employ generically, although by some it has been temporarily preempted for the fusiform bacillus and the so-called spirochaetes found in Vincent's Angina. This disease and many other related ones will fall within the scope of the series of studies being at present conducted, which it is hoped will prove advantageous to a more fundamental understanding of their bacteriology.

CASE HISTORY AND SOURCE OF THE CULTURE

In the summer of 1916, J. B., sixteen years of age, underwent an operation for appendicitis. The latter condition was found to be complicated with an abscess, which was drained. Convalescence occupied a period of about six weeks. Although the wound healed, the patient did not regain his health, as was manifested by a continuous fever of

¹ This paper was presented before a joint session of the American Association of Immunologists and the American Society of Pathologists at their Annual Meeting, March 28-31, 1917, in Philadelphia.

from 100° to 102°. In March, 1917, a futile exploratory abdominal operation was performed, the symptoms directing attention particularly to the right hypochondriac region. The temperature fell to normal for a few days, following the operation, but soon rose to 103°–105°, after which very marked daily variation became the rule. In the morning it was usually normal and at times subnormal, only to be followed by a marked afternoon rise of 103°–105°. At this time pus and albumin were found in the urine, a polymorphic leukocytosis of 17,000 to 20,000 was present, and a bronchial catarrh, which had been noticeable from the first, assumed a more severe character.

Prolonged search of the mucopurulent sputum revealed a few small, opaque, white spherules about 1 millimetre in diameter. They were not at all caseous, but were quite cohesive. They reminded one of the sulphur granules of actinomyces infection. The sputum was rich in lymphocytes. When the spherules were crushed they were found to consist of necrotic masses and a thread-like branching micro-organism apparently in pure culture. These organisms were non-acid fast and Gram-negative. Several months later samples of sputum showed the same cohesive granules to consist of interlacing, very slender filamentous forms which were made up apparently of single rows of tiny granules. They stained poorly in Loeffler's, but very well in fuchsin. Among them were found also some vibrio forms and fusiforms.

On March 28, 1917, a lung puncture was made at the ninth right interspace, in the post-axillary line, at the point of greatest tenderness. A slight amount of serum and flaky material was obtained, resembling the spherules found in the sputum. Examination of the material showed it to consist almost entirely of bacterial growth. There was marked pleomorphism of the elements found. Asymetric bacillary or filamentous forms combined with large circular or ovoid forms predominated. The former varied from 5 to 20 microns long, the latter from 2 to 4 microns in diameter. These circular forms simulate the large giant cocci to be described in the first blood culture. There were also many thin wavy filamentous forms. These organisms grew only anaerobically. In broth the circular forms gradually disappeared leaving filamentous and coccal forms. The latter varied in size from about 0.25 microns to 1 or 2 microns. They usually occurred in groups. Repeated anaerobic plating showed that this culture was probably pure despite the confusing variety of forms.

On April 7, 1917, a second abdominal operation was performed, revealing a renal abscess, the pus from which was aspirated and a piece

of the kidney removed for histologic examination. The pus contained curved bacillary forms, occasionally faintly granular and not infrequently branching. They were frankly Gram-negative and in Loeffler's stained poorly. In Romanowsky they stained deeply blue. There was also present an occasional small group of metachromatically staining, extremely small coccil and diplococcal forms. This pus yielded absolutely no growth on any medium under aerobic conditions. On blood agar, anaerobically after nine days, small elevated colonies appeared. They had precisely the morphology of the Klebs-Loeffler bacillus, but emitted a putrid odor characteristic of *Bacillus fusiformis*. After three days the same pus on hydrocele agar developed greyish elevated colonies of a granular bacillus with pointed ends, which also developed this putrid odor (fig. 1). They had the staining reactions of *B. fusiformis*.

These organisms differ somewhat from the fusiform bacilli usually seen in exudates. Although many have the chromatin disposed as a single granule or as two granules paracentrally located, others show it disposed in rod shape, resembling vibrios. The latter may be S-shaped or straight. This culture stained in Romanowsky showed a delicate faintly staining substance surrounding the bacteria, in mantle-like fashion. It is in reality a periplast, which probably originates from the centrally located vibrio-like chromatin. Forms simulating these have been grown in association with the typical filamentous form and will be discussed further on page 522 (see fig. 2a).

The architecture of the kidney was almost entirely displaced by granulomatous tissue, characterized by fibrous change, and a diffuse infiltration with a large mononuclear type of cell answering the description of the endothelial leucocyte. In many places the infiltration seemed to be made up almost entirely of these cells. Typical plasma cells and polymorphonuclears were also present, the latter being most conspicuous in the remnants of the uriniferous tubules and glomeruli. The histopathology was suggestive of the actinomycotic lesion, but lacked the characteristic colonies. An examination for tubercle bacilli was negative, but a Gram-Weigert stain, cautiously decolorized, revealed most of the forms found in the smears of pus.

On December 7, 1917, one week before the patient's death, he developed multiple pleural sinuses from both pleural cavities. A sample of pus from each side stained in Romanowsky shows the variety of forms already described. There were large numbers of true fusiform bacilli, the giant coccus forms with peripheral granules, the small oval eccen-

trically granulated forms of various staining intensities, and the very thin wavy pink staining forms, some of which can be seen springing from the end of thicker, blue staining vibrio forms. There are also found the interrupted forms—slender curved bacilli in threads but connected by a non-staining link of appreciable length. All the discharges had the characteristic putrid odor of infections with *B. fusiformis* and the lesions were of a sloughing, gangrenous character, as was instanced by the breaking away of the wound stitches, due entirely to the direct but indolent extension of the process into the healthy tissue that held them. All attempts to obtain an autopsy on this patient were unfortunately futile.

The wide morphologic diversity evidenced by both the tissues and cultures favors the interpretation of a mixed infection, yet a careful analysis of the findings, especially the results of the blood cultures, revealed phenomena very inadequately explained by this hypothesis, and led me to seek a more satisfactory one. In the first place, the anaerobic character of all flora obtained from closed lesions (except the blood) was to say the least unusual, in consideration of the marked pleomorphism of the flora of the individual lesion. Most of the forms found were known to be rather closely related biologically. It is of note that the odor permeating the patient's room following the abdominal operation could be compared best with one thing, namely, a culture of *B. fusiformis*. Likewise the culture of *B. fusiformis* isolated from the renal pus as well as the aerobic coccus forms growing from the blood and from the Berkefeld's filtrates of the blood-serum produced this same odor, but the aerobic cultures lost it after the third generation. The fact that the aerobic coccus and the anaerobic bacillary form possessed this biologic character in common also suggested relationship between them.

The clinical course of the disease was akin to that of tuberculosis, or some mycosis. The intermittency of the clinical symptoms was most noteworthy, the patient having experienced about half a dozen clinical cures before he actually died. For weeks at a time he would have no fever, and would gain greatly in weight and strength. Assuming that the condition was mycotic in nature, it seemed reasonable to consider the various

morphological forms as phases in the life history of one organism. Such an hypothesis was particularly serviceable in the interpretation of a very peculiar, and in my experience novel phenomenon, observed in connection with the blood cultures. In its explanation the mixed infection idea was inadequate, and its investigation forms the starting point for the entire study.

BLOOD CULTURES

Ten cubic centimeters of blood were first grown in 50 cc. of broth in two separate flasks of the same medium (broth 8). After forty-eight hours a small amount of granular sediment appeared, but the supernatant was entirely clear. Examination of one flask at this time showed some very large circular forms (giant cocci, fig. 3) varying from 3 to 4 microns, staining faintly or not at all in Loeffler's and negative to Gram, except some granules at the periphery of the cell.² Wright's stain as well as cresyl blue were used to differentiate these from erythrocytes or their products. With Romanowsky they stained quite satisfactorily.

Until the fifth day the growth consisted of a slowly increasing, coarsely granular sediment. At this time, however, reproduction occurred with such amazing rapidity that the culture became diffusely clouded in three hours' time, which event constitutes the novel phenomenon to which I have referred. To my surprise the culture then contained a preponderance of usual-sized diplococcus and coccoid forms. The same findings were

² At this juncture 2 cc. of the broth (no. 8) were pipetted off this flask and placed at room temperature for a day and then in the ice-chest. In three days, long branching filaments developed, some taking their origin from the intracellular coccoids, others apparently arising from the peripheral granules of the giant cocci (figs. 4, 5, and 6). This culture has been carried for more than 100 generations under varying conditions, but diplococci such as germinated in the flask on the fifth day have never been separated from it. As will be seen, steps were taken to rule out contamination in this instance, and such response to environment is one of many observations favoring the cyclic nature of the changes seen. The cocci developing from the blood in another batch of broth, although suggesting filaments by the short spicules that arose from some of them, would without other evidence have been misleading.

present in the second flask which had not been exposed to the air by examination of it. A second blood culture taken in another flask of a different batch of broth (no. 16) developed these same diplococci and coccoid forms in twenty-four hours. Reference to the media book showed certain irregularities in the preparation of the first batch of broth. In order to ascertain the possible influence of this factor, a third culture of 10 cc. was taken and equally distributed between flasks of broths 8 and 16 with results which are a repetition of those just related. It seemed certain that the broths were quite different, and certainly possible that this difference found expression in the way just related.

Warm stage studies, the results of which will be described presently, suggested that the organism was present in the blood serum in filtrable form. Accordingly 5 cc. of freshly drawn citrated serum was diluted with 25 cc. of sterile NaCl solution, and passed through a Berkefeld N. filter. The filtrate was mixed with the no. 16 broth with the consequent development of the same diplococcus and coccoid forms found before. The luxuriantly growing diplococci were found pure on plating. Further evidence for the existence of the organism in the blood in filtrable form is seen in the fact that a suspension of these diplococci, grown as such, refused to pass the same Berkefeld filter that had previously passed the filtrable stage of the organism through its pores. Furthermore, the identical filter, after being cleaned and sterilized, once more passed the original sample of diluted blood serum and gave rise to the same cocci. The experiment was repeated in order to be sure that the refusal of the cultured diplococci to pass the filter was in no way dependent on a mechanical plugging of its pores during the first experiment. These experiments would seem to show that there existed in the blood serum filtrable forms which germinated into diplococci and coccoids, which forms themselves were not filtrable. But I wish to emphasize the fact that the experiments do not prove that these diplococci and coccoids may not give rise to another order of filtrable body, conditions for whose germination have not been fulfilled. The evidence for the exist-

ence of different orders of gonidia will be discussed later. The filtrable bodies I shall hereafter speak of as gonidia. Entirely in line with their presence in the blood is their microscopic appearance in the pus of the renal abscess. Stained in Romanowsky, they appear as groups of very small pyriform structures metachromatically staining. They are also found, together with the coccus and coccoid forms, in the pus as well as in the tissue of the renal granuloma itself.

A more detailed study of the provisionally designated giant cocci, already referred to as occurring in the blood culture, shows that some of them contain from four to six distinct oval intracellular bodies, similar in size and morphology with extracellular forms of the same size (fig. 5). These latter constitute part of the coccoid forms and are to be distinguished from the typical diplococci and the gonidia. In addition to the oval intracellular bodies, very many of the giant cocci average four chromatin granules, located usually at the poles of the cell.

This latter form has occurred frequently in generations far removed in number from the original blood culture. With Romanowsky the granules stained a deep blue, and were usually clearly defined, while the remainder of the cell was either achromic or varied from light pink in the younger forms to a magenta or frank blue in the older ones (figs. 4 and 7). This staining intensity increased first in the periphery of the cell and was often an indication that the filamentous phase of the organism had been reached. In fact, it can be shown that this peripheral staining intensity often appears to represent the development of intercommunicating filaments between the granules, which later may encroach on the central pink portion until ultimately the whole cell is intensely blue (fig. 4b). Under certain conditions the peripheral granules may develop lateral projections, resulting in a stellate form (fig. 4a). The cell body appears to disintegrate later, leaving the pleomorphic, deeply stained granules attached to long wavy filaments. These later may break up into vibrio and then small coccus forms. Under appropriate conditions, these coccus forms seem to fuse into an amorphous, granular mass, and giant cocci, having the character of many of those

found in the blood cultures will again appear, and the cycle start anew. In fact, the observations on the living forms, as detailed below, were seen in a transplant from such a culture.

WARM STAGE OBSERVATIONS

This method of study was adopted as constituting one of the most direct avenues for the solution of the problem created by the fulminating rapidity which characterized the advent of diplococcus forms into the broth culture. Figures 8 to 11 inclusive show that the giant cocci develop quite definite intracellular changes, the end result of which is the formation and liberation of gonidia. Inasmuch as this entire change occurred in less than half an hour, it might serve to explain the phenomenon above referred to. It seems probable, not only from direct observation but also from the study of stained specimens, that the oval intracellular forms, having on their central end the suggestion of a filament, gave rise to the extracellular forms by a process resembling schizogony among the protozoa.

It is important for a study of these forms that cultivation be carried out in serum or hydrocele broth, as the forms develop slightly if at all on solid media. My preparations were made by pipetting off some of the sediment to a perfectly clean slide, and covering with a cover glass, scrupulously clean, in such manner that no bubbles of air were enclosed, and that no excess of liquid projected from the edges of the cover slip. An airtight cell can then be made by rimming the edges with paraffin, and the preparation usually lasts at least twenty-four hours, if properly made. This is an old and well known method and for this purpose is much superior to the hanging drop; unless the preparation is free from air bubbles, however, much difficulty will be experienced in the necessary stability of the object to be studied. It should not be imagined that these changes can be seen on any occasion or under any conditions. One may search many hours, even days, in properly prepared specimens containing many large forms, before observing clean-cut and definite changes. And when they do occur, they may be of an abortive nature, as indeed happened twice in the specimen described, before the actual cycle depicted was finally consummated.

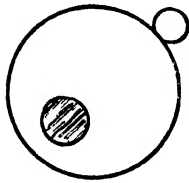


FIG. 8A



FIG. 8B

FIG. 8A. GIANT COCCUS MEASURING FROM $3\frac{1}{2}$ TO 4μ AS SEEN AT 9.00 A. M.

The highly refractile, finely granular central body changes location rapidly, and is actively amoeboid.

FIG. 8B. REPRESENTS THE IRREGULAR, STRUCTURELESS FORM OR RESTING STAGE, FOLLOWING 2 STAGES OF CYCLIC ACTIVITY COVERING PERIOD FROM 9.00 A.M. UNTIL 5.00 P.M.

Resting stage lasted until 10.45 A.M.

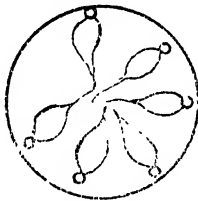


FIG. 9A

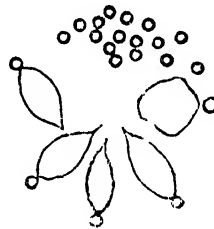


FIG. 9B

FIG. 9A. 10.45 P.M. CELL BECOMES CIRCULAR AND DEVELOPS A DISTINCT WALL

Six oval, highly refractile bodies appear, having a wavy filament on one end and a minute, highly refractile granule on the other. The latter look toward the periphery, the former toward the center. Simulates a protozoal rosette.

FIG. 9B. 11.00 P.M. CELL INCREASES IN SIZE AND WALL BECOMES INDISTINCT

Apparent extrusion of peripherally located granules. Apparent incomplete fusion of 3 oval bodies with resultant gonidial formation.

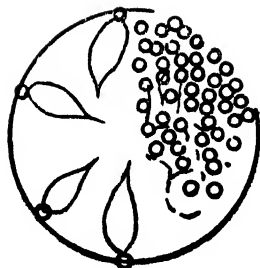


FIG. 10A

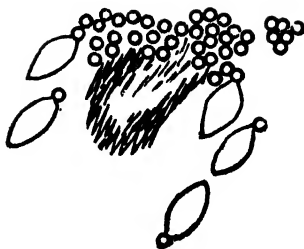


FIG. 10B

FIG. 10A. 11.10 P.M. CELL FURTHER INCREASES IN SIZE

Wall becomes more indistinct, and cannot be made out in the neighborhood of the granules which have increased in number and are actively motile.

FIG. 10B. 11.15 P.M. RUPTURE OF CELL WALL WITH ESCAPE OF GONIDIA

A definite rent appears to three-fourths of depth of cell. Gonidia have rapid oscillatory motion which imparts a movement to the entire cell. Its amplitude of vibration is increased as they are freed into the surrounding medium.



FIG. 11A



FIG. 11B

FIG. 11A. 11.20 P.M. CELL MUCH DIMINISHED IN SIZE, AND OVAL BODIES ARE INDISTINCT

FIG. 11B. 11.30 P.M. THE CELL WALL IS NOW INTACT; CELL IS IRREGULAR IN SHAPE

The oval bodies are imperfectly made out, and little differentiation is present. Cell slowly increases in size until 11.45. It again goes into a resting stage.

Closely related to these forms is another large circular form, usually unstainable, and consequently studied to best advantage in the fresh state. Such forms have been observed under the following conditions: The Berkefeld filtrate of the patient's blood serum developed coccus forms on blood agar, growing readily under aerobic conditions. This culture, after several months' residence in the ice box refused to grow on solid media at 37°. Examination of it microscopically showed what resembled a metachromatic mass of granular debris. When planted at room temperature in plain broth the typical filamentous form of the organism developed, but if planted in broth containing a little hydrocele fluid at 37°, there appeared large coccus forms, which under the warm stage developed cyclic changes similar to the ones just shown. When a drop of this sediment was examined directly in an air tight cell, most remarkable forms were seen. The predominant form is hyaline-like, varies in size from ultramicroscopic to 1 or 5 microns in diameter, and has a clean cut circular appearance. I have observed this form in many cultures under a variety of conditions, and its appearance seems to occur most commonly after apparent fusion and disintegration of the bacterial cells. They are indistinguishable from those adequately described by Hort (1917) in his studies on the meningococcus, although he regards them as ascospores on account of the endosporulation and gemmation and even segmentation which he has observed in them under the warm stage. They certainly give the appearance of reproduction by budding as well as by the schizogony-like process suggested by figure 5. So far, I have not satisfied myself conclusively that gemmation takes place, but I believe it is probable. As Hort has pointed out it is absolutely essential that one study these forms in broth cultures and with wet preparations. To attempt to gain an adequate idea of them with ordinary stains and the use of solid media is to be assured of failure.

I should like to speak of one other morphologic change which is of singular importance regarding the conception of the bacteriology of Vincent's angina and related affections. The symbiosis of *B. fusiformis* and a spirochaete has had traditional

etiologic acceptance. Since 1900, however, there have been a few dissenters, most conspicuous among whom have been Tunnicliff (1905). She contends that the spirilla and *B. fusiformis* are different phases of the same thing. Her principal evidence for the contention lies simply in the fact that in the cultures of *B. fusiformis* certain spiral forms appeared which she feels are identical with the spirochaete of Vincent's angina; but, from her photomicrographs of them as well as from the text, I am inclined to class them among the filamentous and spirillar forms which I have just described. Their width compares favorably with other spirilla and they stain intensely in Loeffler's methylene blue and other simple stains. Such characteristics do not belong to most spirochaetes, including those forms mistaken for such in the ulceromembraneous form of Vincent's angina. These latter stain pink or bluish pink in Romanowsky while both the fusiform and vibrio and spirillar forms of this condition stain a frank blue. We have been able to develop repeatedly in culture the true spirochaete-like forms having the specific staining reaction of the real spirochaete (fig. 12). It goes without saying that they are not genuine spirochaetes; at least I have never observed motility in them. These arise most commonly as lateral or terminal outgrowths from the branching or filamentous form, although more rarely they may spring from the fusiform bacillus itself or they may, under certain conditions, come directly from peripheral chromatin granules of the giant coccus forms. I am not aware that these very slender, wavy forms with the specific staining reaction have ever before been reproduced culturally, from organisms of this class.

Although true spirochaetes may be cultivated from Vincent's angina, as shown by Krumwiede (1913) and others, I do not feel that there is sufficient evidence for regarding them as representative of the majority of the wavy forms found in this condition. Furthermore, by no means all of these forms are motile. There are cultural reasons, as Noguchi (1917) has suggested, for regarding these wavy forms as closely related to the spirilla.

In some experimental subcutaneous and intramuscular ulcers I have observed large numbers of these slender pink-staining

forms, in no way morphologically distinguishable from those of Vincent's angina. They could be seen at times arising from the end of the bacillary form (either diphtheroid or fusiform), and were most numerous when the lesion was nearly healed. It is my contention then, that there are found in these clinical conditions four principal forms: (1) *B. fusiformis*; (2) coarse, sometimes branching, loosely wavy long spiral forms, staining readily; (3) true motile spirochaetes and (4) forms strikingly like spirochaetes in morphology and staining reactions but non-motile, yet easily distinguished from the coarse wavy forms from which they may at times arise, as has been shown in culture. In part from this cultural and experimental evidence, and partly from the fact that in the lesions of the case under discussion spirochaetes were not demonstrated, although fusiform bacilli were cultured from the gangrenous, putrid smelling lesions, and in part for reasons yet to be presented in the study of these organisms, I do not feel that we have adequate evidence for placing a higher etiologic value on the true spirochaetes associated with the fusiforms than we do on the hemolytic streptococci, which may also be associated with them.

GONIDIAL FORMATION

I wish now to present, in brief, evidence tending to show that this strain produces at least three distinct orders of filtrable bodies, and that each form is chiefly, although not wholly derived from a distinct morphologic phase in the cyclic development of the organism. We are in possession of considerable evidence that the granular diphtheroid-like phase develops within itself minute filtrable gonidia, conclusive data regarding the fate of which must be left for future study. The presence of occasional refringent areas located between the staining granules of *B. fusiformis* has been noted by various observers. To a lesser extent these areas are present in diphtheroid bacilli. In the diphtheroid phase of this organism, as well as in other known diphtheroid bacilli, I have observed, under certain conditions, a very marked increase of highly refringent minute circular

bodies, developing in the lighter spaces between the stained bars of the organism. With respect to *B. fusiformis*, it has been stated that the areas are not circular; but, during periods which I wish tentatively to style as gonidio-potent, these spaces seem to be the anlage for the formation of definitely circular bodies.

It is of note that, coincident with a marked intracellular increase of such bodies, very large numbers of similar structures are found free of the bacilli (fig. 13). When growing aerobically, they usually occur in young cultures (six to eight hours) in largest numbers. They appear to stain better as they age, the dye being absorbed first at the poles and on the periphery of the body. They may appear in pairs when free. They may be seen at times on the ends of the organism, and not infrequently bulge from the side, clostridial-like. These considerations suggest strongly the intracellular origin of the extracellular bodies.

Subcutaneous injection of the filtrates into animals has shown results under certain conditions, and more extensive work along this and cultural lines must be done before the nature of these bodies is finally determined. Attempts to grow them have apparently developed forms which appear as very small diplococcoid bodies (stained with Romansky) as well as forms resembling the stellate and giant coccus forms already described. Fig. 14 represents one of the forms, and simulates closely those seen under the warm stage. It is unfortunate that, owing to interruptions in the work, this particular form died out after being laid aside for some months in the ice chest. From their appearance in young cultures, together with their other characteristics, I am inclined to regard these minute bodies as regenerative units, rather than products of degeneration. It seems possible that this process may occur frequently in these organisms, but its extent is greatly influenced by external conditions.

It is not possible at this time to formulate any set of conditions that will with any degree of certainty bring about these periods of gonidia-potent activity. They have occurred under quite diverse and often fortuitous influences apparently, such as the slow evaporation of NaCl solution from a blood agar slant and following transplantation from the depths of an agar shake culture on to a moist blood agar slant.

The French authors, Besson (1913) and Vincent (Besson, 1913), state that these bodies are not spores, inasmuch as they do not have the staining reaction of spores. Tunncliffe (1911) has described spore formation in fusiform bacilli. A personal communication from her indicates that she has based her contention on staining reactions alone, and adds that the bodies may not be true spores. I have noticed a tendency in these granules to retain the stain after decolorization, which has also been shown to be the case with certain diphtheroid bacilli (Mellon, 1917). Although I have made no studies of the tubercle bacillus with respect to the well known granules of Much, the description of the latter inclines me to regard them as comparable to the forms under consideration. These bodies have reacted negatively to the few physiologic tests I have performed with them.

The second order of gonidia is produced chiefly by the filamentous branching forms. They arise either from the end of a main stem or its branches, or directly from the side of either with almost equal frequency (figs. 15 and 2). They stain much deeper than the rest of the cell, and are not resistant to the ordinary stains, as is the first order. Their number depends somewhat on the length of the filament; but, generally speaking, they are more numerous than those of the first order. When a suitable culture was filtered, a finely flocculent precipitate formed in the broth at 37° and at room temperature, which microscopically contained bizarre shaped, irregularly staining bodies, scattered among what was apparently granular debris. Control tubes of this broth did not precipitate, and the nature of this amorphous material cannot be interpreted at present. There is no reasonable doubt concerning the microbic nature of the forms found, as they stain very distinctly; yet, from the fact that they have not yet been successfully transplanted, even though derived from a rapidly growing filament, it is evident that there is much to be learned about them. A variety of forms are discernible. Some are undoubtedly rod forms with rounded ends which are often slightly granular. Others have a diplococcoid appearance, while still others are bizarre shaped,

reminding one of the club-shaped forms of the diphtheria bacillus. It is of interest that none of these forms resemble in the slightest the branching filaments from which they are presumably derived. Fig. 16. Rosenow and Tunnicliff (1913) described small coccus-like bodies in culture from a case very similar to this one. They believe that these coccus forms came from the dilated end of the filaments. They were not able to cultivate them separately, but say they resemble the spores described previously by Tunnicliff. They offer no further suggestions regarding their nature.

The third order of filtrable body I have already described on page 510. They were obtained from the blood serum and also in the third generation of the first blood culture. From the fact that I have not been able to obtain them from any subsequent culture, it is probable that their development in such an early generation is explained by their being carried directly from one culture to another.

Although these orders of gonidia as outlined have developed in connection with these various phases, it is by no means certain that they are always formed in the same way or that bodies answering their description are always seen with the forms described. For example, I have observed occasionally small oval staining bodies in the filamentous forms, and easily staining circular forms on the ends and sides of the granular bacilli. With little regard for size or shape, all spheroidal bodies found in connection with these filamentous forms have received the designation of coccoids. From their difference in behavior it seems probable that there may be a number of bodies of different nature included under this term; and, although it is difficult to speak with assurance regarding all of them, there is a reasonable degree of evidence against all of them being involution or degeneration forms.

CULTURAL AND MORPHOLOGICAL CHARACTERS OF THE DIFFERENT FORMS

The filamentous form occurs with or without branches, varies from 0.5 to 1.5 microns in width, and, in length, from short forms up to those covering one or more fields of the microscope. Under certain conditions large oval knobs may develop at the end of the branches. In common with the filaments, these bodies stain readily and are Gram-negative, and not infrequently contain one or more highly refringent minute bodies, which at times bulge out from their margins. They may occur independently of the filament in which case however, there is usually a short spicule or outgrowth from one or both ends. Optimum growth occurs at room temperature, proceeds slowly in the ice-chest, and may or may not take place in the incubator. The latter temperature seems to conduce to the formation of the terminal oval bodies above mentioned (fig. 6). Beef-heart media seems to be another of the probably large number of factors contributing to the development of these forms.

On slants, growth is luxuriant, the colonies being usually discrete, and after twenty-four hours developing a lemon-yellow pigment on most media. The colonies are raised, moderately moist, fairly adherent, often to the extent of giving the colony a countersunk appearance. In the older colonies, especially, a concentric striation, suggestive of actinomyces, develops. On old, slowly developing slants, coalescence of the colonies is common, and the filaments often fragment into shorter bacillary forms and into diplococci. In transplants the growth is usually filamentous, and it can be said with certainty from warm stage observations that the diplococcus and bacillary forms return directly to the filamentous form on transplantation. In moderately old cultures some of these filaments may lose their staining power and become stippled with fine granules. Similar granules may be seen free of the filaments, and should not be mistaken for gonidial granules, which they resemble closely. Unlike the latter they are not filtrable and are involutionary in nature.

The granular or diphtheroid forms have the characters that are associated with most diphtheroids. They are Gram positive, do not grow perceptibly at room temperature, but in the incubator grow on blood-agar slants, appearing as moist small semi-transparent, discrete non-adherent colonies. In the ice-chest, as a rule, growth is not perceptibly increased; but on some media the whole growth may become changed to large coccoid and diplococcoid forms. In broth, a moderate sediment precipitates from a slightly diffuse supernatant, the quantity of which is increased by the presence of serum. Gelatin is not liquefied. This form is a facultative anaerobe.

The diplococcus form, which may also be arranged like staphylococci, grows luxuriantly on any medium, and its optimum temperature is 37°, although it may grow at room temperature. The growth is luxuriant, white, opaque, moist, confluent and non-adherent on solid media, while in liquid media it grows rapidly and diffusely. Funnel-shaped liquefaction of gelatin stabs takes place along the entire line of the inoculation. It has but slight anaerobic tendencies.

In addition to these forms, all of which grow aerobically, is the anaerobic fusiform type, which is to a slight degree a facultative aerobe. On page 507 I have referred to the periplast formation in connection with these forms. Stripped of their periplast, some of these forms would appear as simple vibrios or filaments. I have encountered, in a culture of the latter, large numbers of what at first appeared to be plump, faintly staining bacillary forms; but more careful study showed many of them to be traversed longitudinally by a filament, or else the latter coincided with the margin of the large bacillary-like form (fig. 2). These forms also occurred at the ends of long filaments, giving the impression that they developed there much in the same way as the larger clubs of actinomyces develop. It will be recalled that the latter have the same general arrangement that I have described for these forms, and I think it fair to regard them tentatively as homologues of the actinomyces clubs. The latter, with the exception of those described by Smith (1918) have never been cultivated, and have been regarded

as a degeneration product of the filament, brought about by reaction of the tissues. In this connection it is of interest that these forms developed in the depths of a culture of "hormone agar," the essential feature in its preparation being the preservation of certain growth products of the tissue employed. The forms did not grow in transplants, and have been encountered inconstantly.

No transition changes have been observed with the fusiform organism found here; hence nothing can be postulated definitely in respect to its relation to the filamentous form. However, Tunnicliff's (loc. cit.) contention regarding the phasic relations of *B. fusiformis* and certain spirilla is suggestive in this connection. Moreover, I have recently encountered true branching in a strain of *B. fusiformis*, which more than ever is coming to be regarded as very closely related to the streptothrices.

INDUCED CHANGES OF PHASE

a. Bacillary to filamentous

It is obvious that, in order to establish the theory that these various forms are phases or stages in the life history of a single organism, it will be necessary to trace the mechanism of transformation of one form into the other. A pure culture of the bacillary form, grown on a blood agar slant, was washed down with sterile broth and distributed over another slant of the same media with a Pasteur pipette. After thirty-six hours at 37°, it was put in the ice-box and, after a month, examination showed a tendency to adhere to the medium. Microscopically, the culture showed coccoid bodies, very variable in size, some being as large as 3 microns. Many diplococcoid forms were present, but only a few of the original granular forms could be seen. Such coccoid changes in diphtheroid cultures are among their best known features.

When these coccoids were sown in broth 8, they developed one or more projecting spicules which, in a few days at room temperature, developed into filaments. Morphologically, no indication of this change was observed among the coccoids of the

blood agar slant at this time. The latter was sealed and returned to the ice-chest for six months longer, and at the end of this time the growth seemed still more adherent than before. Microscopically, many of the large coccoid forms and diplococci showed one or more delicate projections, occasionally branched and of variable length (fig. 17). An occasional bacillary form could also be seen with a long terminal filament projecting from its end (fig. 18). Transplants on blood agar of this culture developed the typical branching filamentous forms. They grew best at a temperature slightly above the room, but also developed in the incubator and at room temperature.

A second culture, having precisely the same origin as the above, after remaining in the ice-chest for the same length of time and on the same media, was transplanted on various kinds of media and under varying conditions, in an effort to regain the bacillary form. All attempts over a course of two months were futile, and it seemed reasonable to assume that the culture was dead; but a transplant on blood agar that had stood at room temperature for a month developed the typical filamentous form. The only varying factor in the treatment of these two transplants from an identical source had to do with the fact that the latter was made in the usual way with a wire, while the former was made from a broth suspension, with a pipette. Still a third transplant made with a wire, the origin of which was in common with the two foregoing, refused to develop filaments after one month's stay in the ice-chest when treated in the same manner as the first transplant, but at a later period developed them when treated in a manner entirely different from the other two. These experiments tend to show the obstructions to any precise formulations of a procedure of this kind, theoretical discussion of which will be undertaken presently.

The mechanism for the development of the filaments seems perfectly clear. The demonstration of their origin directly from the bacillus, as well as from its coccoid forms, and their subsequent cultivation as such, seems to rule out both the contamination and the mixed biotype objections. It is noteworthy that thus far it has not been possible to grow the bacillary form

when the culture had changed sufficiently to make transplantation of the filaments possible. In one instance, the change occurred in ten days, yet the bacilli failed to grow in transplant. In control cultures where no such change had taken place, they grew readily, even after much longer intervals.

As I have already noted, the bacillary form has died out, which fact constitutes the only reason why attempts to confirm these results were not made with a pure line, i.e., a culture started from a single bacillus. Yet it is obvious that if gonidia are actually associated with this form, the purity of the line would still be somewhat in doubt. In the many observations that I have made of the pleomorphism of the branching form, I have never observed the large coccoids developing at low temperatures, such as occurred with this diphtheroid form. On the other hand, the filaments almost invariably developed very small diplococci, as involution forms, both in the ice-chest and at room temperature. It is beyond question that slender wavy filaments do develop from diphtheroid forms at times (Mellon, 1915), as well as from *B. fusiformis* (Tunnickliff), but their significance has been an open question, and cannot be considered settled yet. Of interest also in this connection is the demonstration of filaments in the blood of a patient suffering from a severe fusiform infection (gangrenous balanitis) (Mellon, 1919).

b. Coccus to filament

Reference to the blood culture experiments shows the blood serum of the patient was repeatedly filtered through a Berkefeld, and each time, coccus and coccoid forms were obtained. They varied much in size and stained somewhat irregularly, particularly in Gram, where different degrees of reaction to the stain were apparent. Some of the partially decolorized forms contained deeply blue staining granules. Such features in my experience have always suggested that I was not dealing with a stabilized coccus form, but rather with a phase of a higher organism. This culture was repeatedly plated out at 37°, at which temperature it grew luxuriantly; but before a colony was selected

for transplant, it was placed at room temperature for a few days and then in the ice-chest, so as to give opportunity for the development of any mixed forms. This culture was frequently transplanted for ten months on plain agar. It was then planted in an 8-inch tube of no. 8 broth containing some sterile hydrocele fluid. In twenty-four hours, examination of the diffuse growth showed pure cocci. After three days at room temperature, the growth began slowly to change to a flocculent character, at which time the cocci showed a great variation in size, as well as granular staining with slight projections from one or both ends of numerous organisms. At this time, the culture contained numerous non-staining hyaline bodies, some of which had secondary and tertiary circular bodies attached to them, suggesting a budding process. They were best observed when examined in a wet non-stained preparation. Four days later, there were numerous branched filaments in the culture. The original culture of the cocci on plain agar or blood agar continues to grow as such (twenty-four months).

The culture was then transplanted on a solid medium at room temperature in the hope of developing the filaments. After forty-eight hours, nothing but cocci appeared. The culture was then sealed and placed in the ice-box. At the end of four months it showed no macroscopic change, but a microscopic examination gave the appearance of a fusion or degeneration of the coccus forms, poorly defined granules appearing in a non-staining matrix, with Loeffler's blue. Stained in Romanowsky, a great variety of cocci appeared, both as regards size and staining variations. The matrix now stained metachromatically, and was peppered with fine cocci, many of which showed early developing filaments or sprouts (fig. 19). Transplants of this culture on solid media and in broth at 37° gave no growth in a week but, at a temperature between 25° and 30°, developed in three days the typical adherent chromogenic colonies, showing microscopically long branching filaments. Furthermore, all attempts to plate out the coccus form from the filaments have so far been futile.

Inasmuch as neither the filaments nor their involution forms (fine diplococci) were capable of passing my Berkefeld bougies, the only possible explanation of a mixture in this culture seemed to lie in the gonidial granules derived from the filaments. It is, of course, conceivable that they may have been present in the patient's blood. Filtration of the first, as well as distantly removed generations of these cocci, has never yielded a growth of any description, so it would seem that we can dispense with the possibility of a mixture in this instance. However, it should be noted that, of several batches of broth used in these experiments, broth 8 is the only one with which we have been successful in developing the filaments. It is to be remembered that this was the same batch of broth which made possible filamentous development from the blood culture, when other batches gave rise to cocci only. The perfection of synthetic media will, it is hoped, make possible the formulation of conditions, the inconstancy of which has been a formidable source of confusion in studies of this kind.

Associated with these apparently *direct* morphologic transformations, selected because they leave almost nothing to the imagination, are subsidiary ones whose rôle is probably intermediate in nature. Under a variety of conditions and frequently in young cultures, an apparent fusion of the cellular substance of the forms takes place, accompanied by certain staining changes and the development of other forms whose nature is still undemonstrated. These changes simulate what Löhnis and Smith (1916) speak of as symplastic. Almquist (1917) has described similar changes occurring with *B. diphtheriae* and other organisms. They are under further study at present; and, without making any dogmatic assertion regarding their nature, I am sure that the extent and frequency with which they accompany the more direct transformations presages for them a significant rôle in this process.

DISCUSSION

I wish it to be clearly understood that I do not consider these observations as sufficiently complete in themselves to be conclusive proof of the type of cyclic change that I have hypothe-

cated as a working basis. It is obvious that there are many "missing links" in the chain of evidence. Yet the same comment applies in even greater degree if one attempts to explain the observations as a whole in other ways. I am frank to admit that our present knowledge of bacteriological media is not sufficient to enable one to produce some of the reported findings at will; yet it is pertinent that, while certain batches of the media "held out," it was possible to verify them repeatedly.

It is obvious that there are other explanations for the changes described, the commonest, of course, being that the forms are a mixture of different species. Since Barber's invention of a method for the isolation of single bacterial cells, its application in the concrete case has been accepted by many as prerequisite for the demonstration of biologic transformations. It is unquestionably a valuable method in such studies, but, like other single methods, has its limitations, which are more evident to those who have actually used it, perhaps, than to others.

It should be obvious, even to those who sponsor it with a minimum of discrimination, that its usefulness is, roughly speaking, directly proportionate to the size of the organisms which one desires to isolate; and when their size renders them invisible, or nearly so, its categorical quality disappears. It would appear that, to a certain extent at least, these are the conditions prevailing here; and, although the single-cell method is being used where it seems most applicable, its sphere of usefulness can be more precisely delimited only when we learn more of the nature of the filtrable bodies associated with this organism.

There is good reason to believe that otherwise pure cultures of bacteria are often mixtures of biotypes, and such an explanation is usually sufficient in the minds of some to account for *any* experimental transformations. Changes in environment may bring some one of these types into active growth and suppress others, changing very markedly the morphologic picture of a culture.

It is inevitable that this theory of selection should be invoked to explain many phenomena to which it may bear a remote relation, in the same way that Ehrlich's theory of receptors has,

for so many years, retarded advances in immunology. A fair example of such an interpretation is seen in the recent work of Eberson (1918) on the diphtheroid group. The work (Mellon, 1917) which he refutes, is comparable to the subject matter of this paper; yet, approaching it from one angle only, he aligns it with this facile explanation, without even considering the possibility of a cyclic interpretation. In a future communication, it will be possible to focus a more detailed discussion on this point, in the light of this and allied investigations.

It is perfectly obvious that the various phases of this organism are particularly susceptible to changes in environment, yet it should be remembered that the organism itself is not entirely a passive medium. The extent and nature of its response to environmental conditions are functions of its own inherent qualities, which are handed down from generation to generation. However, it is entirely probable that changes may take place in a race of organisms, entirely in accord with their inherent capacity of response to environment, and those changes be so slight as only to become discernible when attempts are made further to modify them in a given direction, which must, of course, be in accord with their capacities for change. Whether we consider such change to occur in a clone, or whether it be the expression of a mixture of biotypes in a culture, bears on our problem only insofar as to require a different combination of environmental factors to evoke the organism's inherent capacities in a certain direction. An instance will point the meaning: reference to the experiments dealing with the development of the filamentous from the bacillary form shows that one cannot always rely precisely on the same means to bring about this result, even though the source of the culture is the same. Even in an organism that has inherent qualities for progressive change, the environmental limits afforded by the usual artificial medium are often inadequate for their evolution. In this instance, the demonstration of filaments arising from the ends of the granular forms leaves little doubt as to their origin.

Furthermore, when cultures become adapted to such conditions, it becomes a matter of increasing difficulty to induce pro-

gressive changes—they become stabilized, so to speak. I have noticed this particularly in attempting to develop the highly refringent intracellular bodies of the granular form; but the filamentous form is more tractable in this respect.

MUTATION

Ordinarily, changes of the sort typified by the bacillary-filamentous transformations would be viewed as mutations, although the whole subject with respect to bacteria seems to lack unanimity of opinion. If we adopt the more conservative idea that mutants only occur under constant conditions, some other interpretation must be forthcoming for these changes, which are to so great a degree a function of environment. Fermentative differences have often been interpreted as evidence of mutation when occurring among the lower bacteria. The literature contains many instances of such in the colon-typhoid group. Hort (1917) has brought to light much evidence for the occurrence of cyclic change in this group, and would in this way explain the so-called mutations. Since reproduction by equal binary fission has always held sway as practically the sole method of bacterial division, the presence of biotypes in cultures could be best explained by mutation; but, if reproduction can occur in other ways, even in a slight degree, the presence of mixed types or variants may have a more simple and satisfactory explanation. The fuso-spirillary organism has the advantage of being on the borderline between the lower and higher bacteria. It is well known that the latter forms have a complex life history, while the so-called branching involution forms of the diphtheria and tubercle bacilli have always suggested close relation with stabilized branching forms.

INVOLUTION AND PLEOMORPHISM: RELATION TO THE WORK OF OTHERS

Finally, it may be considered that this organism represents an example of pleomorphism. This was the explanation advanced for the transformation of certain diphtheroids to diplo-

cocci (Mellon, 1917). Although more marked than the usual pleomorphic changes, they did not seem to merit the designation of the more pretentious process of mutation. Their cyclic nature was suggestive enough to cause this, a similar study, to be approached with those considerations definitely in mind.

Bayon (Besson, 1913) has described an organism from the lesions of leprosy, that has very pleomorphic characters. He says it assumes one of three forms:

(1) A non-acid-fast and non-acid-resisting streptothrix; (2) a pleomorphic, acid-resisting diphtheroid bacillus, and (3) a definitely acid-fast bacillus, indistinguishable from the bacillus in the tissues.

Williams (Besson, 1913), also grew an organism from leprosy lesions, and describes the following forms:

A, on broth media and on potato-broth, a non-acid-fast streptothrix in the mycelial stage which produced acid-fast rods; B, on milk and lemco-broth, a non-acid-fast diphtheroid bacillus which also produced acid-fast rods; C, on Rost's medium, an acid-fast bacillus which is but the broken-down stage of a streptothrix which was cultivated from a leper passed through respectively all the stages described above.

Dick and Tunnicliff (1918) describe an organism (*Streptothrix putorii*) isolated from a case of rat-bite fever, which resembles this one in many respects. It is noteworthy that they obtained a diplostreptococcus from the blood in the first cultures, which agglutinated with the patient's serum in 1:80, while the streptothrix itself reacted in but 1:20 dilution. We have no way of knowing definitely whether this coccus may have represented a phase in the life history of the organism; and I desire merely to mention it as a possibility, there being no good reason why streptococci might not be present in such cases as well as the additional ones to which I am referring. Middleton (1910) and Douglas, Colebrook and Fleming (1918) have isolated respectively a diplococcus and a *Streptococcus pyogenes* from similar cases, and in the latter instance the organism agglutinated the patient's serum in a 1:60 dilution.

Smith (1918) has recently described a pleomorphic bacillus from the lungs of calves simulating actinomyces. This organism seems to have certain features in common with the fusospirillary organism, chief among which is the cultivation of several distinct morphologic forms which he interprets, not as due to a mixed culture, but as having a cyclic relationship. Certain progressive changes obtain in his culture, as instanced by the complete disappearance of the bacillary form and its replacement by a coccus form. These bacilli, when associated with the club forms, also disappear simultaneously with the development of changes in the latter. Again, they have in common the quality that certain forms resistant to culture are developed from actively growing forms. The development in my cultures of forms that may be homologues of the clubs of actinomyces is another point of contact in the studies. Those of Smith's organism are, of course, real clubs, while the ones I describe are much smaller, but apparently of the same respective origin. The organism described by Lignieres and Spitz (Smith, 1918) and regarded by Smith as practically identical with his organism, develops bacillary forms, which later give rise to diplococci and streptobacilli.

Park and Williams (1910) show pictures of diphtheria bacilli, some of which simulate the stellate forms of figure 4. Their description of them betokens the fact that, under the conditions of the experiment, namely, prolonged growth in broth with occasional mechanical disturbance of the pellicle, changes in the reproductive mechanism occur, which suggest to them the process of autogamy and, furthermore that, when the cultures reach this stage, division proceeds *solely* in these involution forms. The fact that similar "branching involution forms" can actually be made to go further and reproduce themselves as branching filaments, is evidence that their observations may admit of a different interpretation in some instances. It would seem that the change produced in the broth by the growth of the organism sooner or later inhibits or prevents development of the organism in its bacillary form; yet its adaptability is expressed in atypical reproductive changes which result in new forms, the latter being the only forms to divide thereafter.

There is no justification at present for saying that all members of the diphtheria group could be made to produce branching filaments, even though the diphtheroid-like phase of this organism in question differs in no essential particular from diphtheroids as we now know them. Still, it should be emphasized that, even as regards the organism with which I am working, it is essential, before the new forms will reproduce as such, that two conditions be fulfilled. The induced changes, typified in part by the above description of Park and Williams, must proceed past a definite point in the evolution of the organism or, on transplantation, the old forms and not the new ones will multiply; secondly, that transplantation must be made to an environment suitable to the newly developing forms, inasmuch as the change in the original medium presumably conditioned their development.

In essence, both the processes of pleomorphism and involution predicate the return of the altered forms to normal on return to "normal conditions." In light of the two considerations just adduced, it repeatedly becomes a question in my mind whether these pleomorphic forms always returned to normal, or whether there occurred a preponderating development of the original forms, the pleomorphic forms either refusing to develop or reproducing so slightly as to be over-looked. The well known selectivity of various media for certain forms would also speak in favor of such a theory.

Furthermore, the apparently lethal effect of residence of cultures in the ice-chest I believe may often be explained in this way: that they refuse to grow, not because they are dead, but because their protoplasm has undergone changes which refuse to respond to the conditions of growth which are not the counterpart of those that have taken place in the organism. I have verified this theory in quite a number of instances when working with organisms of this group; and, without attempting to formulate a rule in accordance with this and associated factors, I may say that temperature changes have been a most potent agency for the induction of evolutionary changes.

RÉSUMÉ

There has been isolated a fuso-spirillary organism from a case in which it caused generalized infection, the point of origin being presumably in the appendix. Its branching filamentous forms relate it closely to the streptothrices, while its bacillary and coccal phases, if we regard them as such, relate it to the lower bacteria. It is noteworthy that the branching filaments were not cultivated from the renal abscess or the lung puncture, although many of them could be demonstrated from the material in both locations—indeed, they constituted the bulk of the flora present in the lung puncture material. They were cultivated from broth blood cultures, however, partly as the result of an irregularity in the preparation of the medium and partly from a radical change in the environmental conditions at a certain stage in the culture's development.

The phenomenon of pleomorphism, undoubtedly well founded, may not explain all morphologic changes in bacteria. Some of those occurring in this strain I am inclined to regard as evolutionary rather than involutionary. As evidence for this view, take for example the demonstration that the filaments originate directly from the body of the granular diphtheroid forms, and can subsequently be cultivated in purity. Furthermore, filtrable forms have been demonstrated in the blood of the patient and in culture, although the ultimate fate of but one order of them has been shown. Warm stage observations have yielded valuable information regarding the origin of some of the filtrable forms, and thrown light on the cyclic activities of the organism. This method is much superior to the single-cell isolation method in a study of this kind, mainly because of the relative invisibility of some of the forms.

I regard the process of phase changes, so to speak, as a function of the environment; but the quality which makes such response possible belongs intrinsically to the organism. Tentatively, at least, I cannot regard these as mutation changes. The latter have been adduced to explain the origin of biotypes from pure lines of bacteria. If one regards mutation conservatively, as of rare occurrence, cyclic changes may offer a better explanation for their origin. Viewed in this light, perhaps some of the

hopeless numbers of bacteria which have received designation as separate species may ultimately come to be considered in some more collective way.

No method can, at present, be accurately formulated for the induction of such changes, not only because there is as yet no such thing as a standard medium, strictly speaking, but even more because of the fact that the stability of bacterial protoplasm is, after all, not absolute. Such lability as it possesses is, however, an intrinsic quality and is not a formidable barrier to the well known quality of cultures to breed true to type—that is, to laboratory type. The transitions as outlined have been repeated many times, but almost never has it been possible to repeat the process in precisely the same way.

Grateful acknowledgment is made to the Directors of this Institution, whose far-sighted policy is responsible for the erection and equipment of an adequate Department of Laboratories, making possible work of an investigative character. I wish also to acknowledge the loan, by the Bausch and Lomb Company, of an apochromatic 2-mm. objective for photographic work, in lieu of one previously purchased but not deliverable until after the war. I wish also to acknowledge the helpful coöperation, clinically, of Dr. S. W. Bradstreet, the physician in charge of the patient on whom the studies were made.

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EXPLANATION OF PHOTOMICROGRAPHS

FIG. 1. Fusiform bacilli grown anaerobically and in pure culture from the renal pus. At *a*, the chromatin has a rod-shaped distribution with a delicate protoplasmic prolongation at either end. Stained in Romanowsky. $\times 800$.

FIG. 2. At *a*, is a form which may represent an homologue of the actinomyces club. Beginning at the left, the deeply stained filament takes a somewhat diagonal course through the periplast, but at the right is marginally located. The faintly staining periplast is divided into three sausage-like loops, the latter of which extends beyond the end of the filament. The remaining two filaments in the field show latterly budding gonidia of varying size. Some larger coccoids are free. Carbol-fuchsin. $\times 1600$.

FIG. 3. The smaller type of giant cocci developing in no. 8 broth blood culture after forty-eight hours. Romanowsky. $\times 800$.

FIG. 4. At *a*, giant coccus forms with peripheral granules one or more of which later give rise to filaments; at *b*, the filaments are further developed. Other smaller coccoid forms of varying size can be seen. These forms have been developed in culture, but represent another type of giant coccus found in No. 8 broth blood culture. Romanowsky stain. $\times 800$.

FIG. 5. In the center of the field is a still larger type of giant coccus found in no. 8 broth, blood culture. Four oval intracellular bodies with an early spicule or filament arising from one of them; it crosses the clear sector of the cell at the lower part of the figure. Figure simulates a protozoal schizont. Romanowsky. $\times 800$.

FIG. 6. Filamentous form showing true branching. At *a*, is seen a group of oval spore-like bodies, simulating protozoal flagellates. Romanowsky. $\times 800$.

FIG. 7. In the upper portion is a group of bodies averaging from four to six, peripheral granules. At *a*, early formation of inter-communicating bands between the granules. Many of the latter are free. The lower portion of the figure shows a giant coccus with two small granules on the periphery. Romanowsky. $\times 1600$.

FIG. 8 to 11. Inserted and described in text.



1



2



3



4



5



6



FIG. 12. Spirochaete-like forms in culture. At *a*, they apparently arise from the bacillary form. Romanowsky. $\times 800$.

FIG. 13. Granular diphtheroid form developing gonidia, many of which are free. At *a*, one is undergoing lateral extrusion. From a twelve-hour blood-agar slant. Specimen overstained in Carbol-fuchsin to bring out the extracellular forms. For this reason little differentiation is seen in the bacillary forms. Carbol-fuchsin. $\times 800$.

FIG. 14. The large pear-shaped body in the center of the field developed in a Berkefeld filtrate of the first order of gonidia. It contains several light oval intracellular bodies and two large chromatin granules. Romanowsky. $\times 1600$.

FIG. 15. At *a*, is a filament showing three budding gonidial granules: one is terminal, the other two lateral. This culture was rich in extracellular gonidia, a few of which can be seen in this field. The result of a Berkefeld filtrate of this culture is seen in the next figure. Carbol-fuchsin. $\times 800$.

FIG. 16. The more intensely staining pleomorphic forms represent the results of germination of the gonidia shown in figure 15. Carbol-fuchsin. $\times 800$.

FIG. 17. Large coccoid forms derived from the diphtheroid phase showing early development of filaments; at *a*, one develops from a granular bacillus. Carbol-fuchsin. $\times 800$.

FIG. 18. Shows the origin of the filamentous form from a typical diphtheroid form at *a*. Diplococci are also present. Carbol-fuchsin. $\times 2500$.

FIG. 19. Early formation of filaments from the diplococcoid phase. Note difference in average size from the cocci of figure 17. Carbol-fuchsin. $\times 800$.



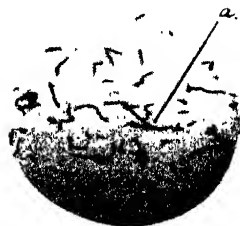
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THE OCCURRENCE OF *BACILLUS BOTULINUS* IN NATURE

G. S. BURKE

From the Laboratory of Bacteriology and Experimental Pathology, Stanford University, California¹

Received for publication, April 5, 1919

Van Ermengem, who was the first to isolate *B. botulinus* from poisonous food and who proved that it was the organism which produced the toxin causing the type of food poisoning known as botulism, made repeated efforts to isolate *B. botulinus* from nature. He made cultures of garden soil, dirt from the streets, mud from ponds and rivers, manure from stables, cow manure, horse manure, duck excreta and the intestinal contents of various species of fish. He was never able, however, to find the bacillus except in two specimens of ham which had caused outbreaks of poisoning (Van Ermengem, 1912).

The work of Kemper and Pollack (1897) who recovered a strain from the intestinal contents of a "normal" hog, is the only recorded case of the isolation of *B. botulinus* from nature. This finding, together with the fact that botulism was at that time chiefly connected with poisoning from sausages and ham, led to the belief that *B. botulinus* was a normal inhabitant of the hog intestine.

In 1917 Dr. E. C. Dickson² of Stanford Medical School, San Francisco, undertook to check up the work of Kemper and Pollack. Dr. Dickson examined the intestinal contents of 250 grain-fed hogs which were slaughtered for market in South San Francisco. The contents of the colon and ilium were the portions chosen for examination. No trace of *B. botulinus* was

¹ Aided by a grant from the State Council of Defense of California.

² Aided by a grant from the State Council of Defense of California.

found in any of the material, showing that *B. botulinus* is not a common inhabitant of the intestines of grain fed hogs.³

Dr. Dickson also collected 10 samples of manure from two groups of garbage fed hogs. Cultures were made and examined but no trace of *B. botulinus* was found.

Dr. Dickson collected samples of soil from two gardens where in the summer of 1917 beans were grown and canned, which caused two outbreaks of botulism in January and February, 1918. The specimens were collected in January and February, 1918, and represented a fair sample of the dirt in the yards, both surface and sub-surface, to a depth of 12 inches. *B. botulinus* was not found in any of the samples.

In the summer of 1918, when I made the survey described in the following pages, there were really no positive data as to the occurrence of *B. botulinus* in nature. There were four localities in central California where outbreaks of botulism from home canned fruits and vegetables had occurred during the previous winter, and it seemed that something should be learned by visiting these houses and gardens during the canning season. In collecting the samples, my object was to cover as wide a range of material as possible rather than to make a complete analysis of any one kind. For that reason this report must serve rather as an outline for further research than as a basis for any very definite conclusions.

METHOD OF COLLECTING AND TESTING THE MATERIAL

The samples were collected in sterile test tubes and petri dishes and reached the laboratory within four days. Sterile instruments were used in handling the material in the laboratory. Cultures were made in a double strength beef infusion broth (Van Ermengem's broth) with 2 per cent glucose and a reaction as near neutral as possible (Van Ermengem, 1912). The broth was oil stratified in the tubes. Witte's peptone was used in order to insure reliable toxin production. This broth medium is particularly favorable for the production of botulinus toxin. All the cultures were made in test tubes unless

³ Dr. Dickson has a report of this work in process of publication.

otherwise stated in the tables. Where flasks were used a large quantity of material was cultured. Just before inoculation all the media were boiled for twenty minutes and immediately cooled by placing the tubes in cold water.

The broth cultures were incubated at 28°C. for from two to six months. They were then tested for toxin. About 10 cc. of the broth culture was passed through a Mandler filter and the filtrate at once oil stratified. One cubic centimeter of this filtrate was injected, subcutaneously, into a guinea-pig. Each filtrate which killed the guinea-pig in forty-eight hours or less, was tested against antitoxins of *B. botulinus* types A and B to determine whether it contained the specific toxin of *B. botulinus*.

For the toxin-antitoxin test three 250 gram guinea-pigs were injected subcutaneously, as follows:

GUINEA PIG NUMBER	FILTRATE	ANTITOXIN
1	1 cc. of filtrate dil. with nor. salt sol. so that 1 cc. kills a 250 gram guinea-pig in about forty-eight hours	1 cc. of antitoxin for <i>B. botulinus</i> type A*
2	1 cc. of filtrate dil. with nor. salt sol. so that 1 cc. kills a 250 gram guinea-pig in about forty-eight hours	1 cc. of antitoxin for <i>B. botulinus</i> type B
3	1 cc. of filtrate dil. with nor. salt sol. so that 1 cc. kills a 250 gram guinea-pig in about forty-eight hours	Control—no antitoxin

* The antitoxins used were of such strength that 1 cc. neutralized 200 minimum lethal doses of the homologous toxin. One minimum lethal dose of botulinus toxin is that amount which will kill a 250 gram guinea-pig in forty-eight hours.

A reliable toxin-antitoxin test may be obtained with a toxin, 1 cc. of which requires four days to kill. With a weaker toxin the antitoxin test is not reliable for in some cases the pigs develop typical symptoms of botulism but recover after about two weeks while in other cases they remain well for a month and then die with typical symptoms after a few days of illness. In

the following charts four cultures are marked as doubtful because the toxin in the filtrate was too weak to give a decisive toxin-antitoxin test although the symptoms of the guinea-pig that died were entirely typical of botulism.

Where a filtrate gave evidence of containing a weak toxin an effort was made to obtain a stronger toxin by making a transplant from the original culture into a fresh tube of "Van Ermen-gem's" broth. All the unfiltered portion of the original culture was transferred into the new tube, which was then incubated for about four weeks and tested for toxin. In one case (Palo Alto culture from a bird-pecked and moldy cherry) I was successful in getting from the transplanted culture a strong toxin which proved to be *B. botulinus*, type A.

The incubation period of from two to six months, was too long, as the average life of *B. botulinus* in broth cultures is about three months. My original plan was to incubate for from six to ten weeks. The longer period was necessitated by delays which were unavoidable. I believe it very probable that if the original plan had been adhered to the percentage of positive botulinus cultures might have been higher, for about one-fourth of the guinea-pigs receiving the initial dose of filtrate, died in from two to six weeks, some especially in the Oakdale series, showing marked symptoms of botulism. All efforts to obtain a strong toxin from sub-cultures failed, except in the one case in the Palo Alto series mentioned above.

SURVEY FOR *B. BOTULINUS*, JUNE, JULY AND AUGUST, 1918

The material was collected from five localities in the central section of California, two of these, Palo Alto and Hollister in the Santa Clara Valley, Oakdale and Madera in the San Joaquin Valley, and Berkeley on San Francisco Bay. The towns were all more than 50 miles distant from each other.

In the Palo Alto series the material had no connection with any previous outbreak of botulism. In the four other localities, however, outbreaks of botulism had occurred during the previous winter, which were caused by home canned fruits and vegetables put up in the summer of 1917. I visited the gardens and the orchard where the vegetables and fruit were grown, except in

Hollister where the peas had been purchased from a peddler. In Hollister and Berkeley an examination was made of the household conditions under which the canning was done. The time chosen for collecting the material corresponded as nearly as possible with the time at which the canning had been done the previous year.

The following is a detailed description of the places and conditions under which the various samples were collected, the character of the samples and the results of the toxin-antitoxin tests. The data are divided into groups according to the places from which the material was collected.

Palo Alto, California

There had been no recorded outbreak of botulism in or near Palo Alto for several years. The corn, beans, peas, peach and cherries which were cultured were obtained in a first class grocery store. All except the cherries were carried direct to the laboratory.

The corn had been set aside as unfit for sale and was badly worm eaten. Four test tube cultures were made of the caterpillars from the corn and five flask cultures of the corn itself. *B. botulinus* was not found in any of the cultures.

One test tube culture each was made of the beans, peas and peach. The peas and beans were dirty and spotted and the peach was partially decayed. The cultures were negative.

The cherries were bought for preserving and were all a little underripe. They were picked over on the unscreened porch of a house in a clean section of the town. There were no horses, cows, or pigs kept anywhere in the neighborhood and dogs were the only animals kept on the immediate place. There were no ants about the house and very few flies. The cherries from which the cultures were made were either slightly bruised or bird pecked. There was no decay or mold on them when they were separated from the other cherries. They were placed in a clean paper bag on the railing of the porch where they remained for two days before they were taken to the laboratory. There

were no spiders or insects in the bag when it was taken to the laboratory but the cherries had molded, wherever the skin was broken.

Nine test tube cultures were made of the cherries. In two of these cultures toxin of *B. botulinus* was produced. One of the positive cultures was made from a bird-pecked moldy cherry, the other from a bruised and moldy cherry. Both contained type A toxin.

Two cultures were made from a caterpillar and four from the crop and gizzard contents of birds killed in the yard of the house where the cherries were picked over. The cultures were all negative.

Hollister, California

The place from which this material was collected was a cross roads saloon about 7 miles from the town of Hollister. It was an exceedingly dirty place. Hogs, cows, and horses were kept in sheds clustering around the back of the house. There was an open privy about 100 feet from the kitchen door. Chickens, mud and manure were everywhere, even on the porches. A leaky water tank was built over a store room adjoining the kitchen. Gray slugs crawled over the walls of the kitchen and store room at night. There were swarms of flies in the house and out of doors.

In the summer of 1917 the housewife canned peas, which she had bought from a peddler. One jar of the peas had spoiled in February, 1918, and the contents was thrown into the garbage which was fed to two hogs. The hogs, one full grown and one small, developed typical symptoms of botulism and *B. botulinus*, type B, was recovered from the stools while the animals were sick. The animals recovered after a few weeks. Three months later, when these specimens were collected, the animals were perfectly healthy, and the small hog had grown to full size. They were later sold to a market.

Cultures were made of the tap water from the kitchen and also of flies collected in the kitchen. In the yard cultures were made from slugs, earth-worms and sowbugs, from chicken

manure, straw and dirt from the chicken yard, from mud under a leaky faucet and from fresh manure collected in the pen of the large hog referred to above.

Toxin of *B. botulinus* type B was produced in one of the five cultures of hog manure. The strain in this culture was of the same type as that isolated from a stool while the hog was sick. It was impossible to make a more complete investigation of the case as the hogs had been killed for market and the pens cleaned up by the time this culture had been tested out. Cultures of all the other material were negative.

Reference has already been made to the work of Kemper and Pollack who isolated a strain of *B. botulinus* from the intestinal contents of a "normal" hog and to the fact that Dr. E. C. Dickson of Stanford Medical School was unable to verify their work although he had examinations made of the intestinal contents of 250 grain-fed hogs and 10 samples of manure from two groups of garbage-fed hogs. The second finding of *B. botulinus*, type B in the manure of the Hollister hog, four months after the contaminated food was eaten suggests very strongly that Kemper and Pollack's "normal" hog may have been fed contaminated food a short time before their cultures were made. Hogs are fairly resistant to botulinus toxin so that there might easily have been no record of the animal's illness.

Oakdale, California

The garden from which material was collected at Oakdale was one in which pole string beans were grown in the summer of 1917. Some of these beans were canned. One of the jars spoiled and was the cause of an outbreak of botulism. In July, 1918, conditions in the garden were practically the same as in the previous year. The section of the town in which this garden was located was quite new. A garden had been made on the place only two years before and no manure had been used. There were no horses, cows, pigs or chickens kept on the place. Beside pole and bush beans, there were tomatoes, wax beans, and melons. The soil was clean sandy loam, well drained. There had been no rain for several months. Irrigating ditches

supplied the water. The samples were collected in the blazing sunlight with the thermometer at 100°F.

Twenty-three cultures were made from spotted leaves and pods of the pole and bush string beans, from ants, spiders, bugs and grasshoppers, collected about the plants and from dirt from the mouth of an ant hill. In one of the three cultures of pole bean leaves spotted by insects or some other small animal toxin of *B. botulinus* type A was produced.

Another culture, containing spiders and small bugs contained a weak toxin. In the toxin antitoxin test the control pig and the pig which received the filtrate botulinus antitoxin type A developed very evident symptoms of botulism about the fourth day but later recovered. As it was impossible to obtain a stronger toxin in transplants from the original, this culture was listed as "doubtful."

Madera, California

The samples were collected from a ranch about 2 miles out of Madera. It was an ordinary small country ranch, with an old house, tank-house, stables and chicken houses. A small orchard of fig and apricot trees adjoined the house on one side and walnut trees bordered the drive and the main road. Alfalfa fields surrounded the place and there were no houses very close by. In 1917 the owners lived on the ranch and kept chickens, turkeys, hogs and horses. There is no record as to whether they also kept cows or as to what sort of a garden they had. In July, 1917, a lot of apricots were canned in a screened summer kitchen. It was windfall fruit and unfit for market. One of the jars of apricots spoiled and caused an outbreak of botulism in which six persons died, February, 1918.

The surviving members of the family moved away and the place was rented to another family. They stayed only a short time. In July, 1918, when I visited the ranch, no one was living there and there were no domestic fowls or animals with the exception of two horses. There were noticeably few birds, bugs or insects other than ants.

One hundred and seventeen cultures were made from material

collected on the ranch. Five cultures were made of tap water from the house. The remaining cultures were made from wind-fall apricots, which were bird pecked, decayed, moldy or ant eaten; from bugs, flies, ants and spiders; from the beaks, claws and alimentary tract contents of English sparrows, linnets, dove and black birds; from dirt and manure collected in an old hog pen and in the chicken yard where all the chickens had died of botulism six months before; and from fresh horse manure.

No positive culture was found in any of the material. One culture of linnets' claws contained apparently a weak toxin of some sort but it was too weak to give a satisfactory toxin-antitoxin test. The control pig and the filtrate-antitoxin B pig developed typical symptoms of botulism on the third day, but gradually recovered while the filtrate-antitoxin A pig remained normal throughout. This was listed as "doubtful" since it was not possible to obtain a stronger toxin from the transplants.

The ranch at Madera differed from the other localities from which I collected material in that there were no human beings, dogs, cats, pigs, cows, or chickens, living on the place. There was no vegetable garden and very few birds, spiders, insects (other than ants), or other small animals. Neighboring ranch homes were a quarter of a mile distant.

Berkeley, California

In August, 1917, Mrs. M. of Berkeley, California, canned some string beans from her garden. She sterilized them by the fractional method. In January, 1918, it was found that four of the seven jars of beans contained *B. botulinus*. The contents of one jar were fed to some chickens and 24 of them died of botulism, "Limberneck." In August, 1918, Mrs. M. had a few beans growing in her garden in almost the same spot where they had grown the year before and she consented to can these for me, as nearly as she could, in the same manner as those which she had put up the year before. In addition, samples were collected from the house and garden.

The house in which Mrs. M. lived was an old one and was situated in a closely built up neighborhood. The yard was an

ordinary city lot, at the back of which were chicken houses and runs. Between the chicken runs and the house was a garden in which was a small patch of bush string beans and also a patch of mint. The yard was dry at the time my samples were collected. The kitchen and screen porch had been made as clean as possible and there were no flies in either place. Mrs. M. was apparently a woman of average cleanliness.

The beans were gathered from the garden about 1.30 p.m. by Mrs. M. and her daughter, and were picked over by Mrs. M. on the screened porch. She discarded all beans that were broken or decayed. Some of the beans which she passed as good had small hard scars or blemishes on the skin. The beans were washed in several changes of cold water under the faucet in the kitchen. The kettles used to hold the beans were clean. After washing, the beans were cut by Mrs. M. with a clean knife and packed in three Mason quart-jars. Mrs. M. said that she had washed the jars and tops the night before in scalding soap suds. In the morning she had rinsed them in about a dozen changes of cold water. They were again wiped out with a freshly laundered towel just before using. The rubbers were new and were washed in cold tap water from the laundry tub on the screen porch before they were put on the jars.

The jars, with tops loosely screwed, were placed in a deep pot of cold water. The water was brought to a boil. After thirty minutes in the boiling water the jars were removed and the tops screwed down. They were then taken to the laboratory and incubated for six months.

Broth cultures were made from the tap under which the beans were washed and from the tap under which the rubbers were washed. Portions of the beans discarded by Mrs. M. were cultured. Cultures were made of dirt from under the bean plants, of snails, slugs, ants, spiders and a caterpillar, and of beans direct from the garden.

In one of the three jars of beans, canned by Mrs. M. toxin of *B. botulinus* type A was produced, and toxin of *B. botulinus* type B was produced in a culture of a small spider from one of the bean plants. The spider was placed in a sterile tube at

once and did not come in contact with anything in Mrs. M's house. The other material was negative with the exception of two doubtful cultures, one of which was from a sowbug, while the other contained earth, web and droppings from a spider tube in the ground under the bean plants. There was apparently a trace of toxin present in the tubes but it was too weak to give a reliable toxin-antitoxin test.

Forage poisoning

I have recently obtained a culture of *B. botulinus* type A from one of four samples of discolored, moldy hay from a large stack on a ranch near Oakdale, California. This hay was suspected as the cause of one of a series of outbreaks of forage poisoning among horses and mules, occurring in the vicinity of Oakdale in January and February, 1919. The spoilage in the hay ran in veins down through the stack and the samples which were cultured were taken from the deeper layers.

The symptoms exhibited by the horses, as they were described to me by Dr. Eddy of Stockton, California, were very suggestive of botulism. The finding of *B. botulinus* in a sample of the spoiled hay is very suggestive. It does not, however, definitely prove that the animals died of botulism as I was unable to carry out feeding experiments to demonstrate the presence of toxin in the hay.

The successful therapeutic use of a polyvalent botulinus antitoxin in cases of forage poisoning will furnish the best proof of the identity of this poisoning with botulism. An antitoxin furnished by the Agricultural Experiment Station of the University of Illinois was used on two horses of the Oakdale series without success. I have recently tested a sample of this serum and find that it contains only type B antitoxin. In my experience *B. botulinus* type A is more common in California than type B.

Graham and Brueckner, who were the first to claim that forage poisoning was caused by the toxin of *B. botulinus*, have a report of some recent antitoxin work in the Journal of Bacteriology, January, 1919.

SUMMARY

A. Two hundred and thirty-five cultures were made from samples collected in five localities in central California, 50 or more miles distant from each other. The cultures covered a wide range of material, including tap water, hay, leaves, vegetables and fruits in various conditions, insects, spiders, sowbugs, snails, and caterpillars, garden soil, manure from horses, hogs, and chickens, and also samples from the claws and beaks, and crop, gizzard and intestinal contents of birds.

B. Seven cultures containing *B. botulinus* were found. The source of the material in these cultures and the type of toxin demonstrated was as follows:

1. Bruised and moldy cherries.... Palo Alto, Cal., Type A
2. Bird-pecked cherries..... Palo Alto, Cal., Type A
3. Pole bean leaf covered with spots or droppings of insects or small animals..... Oakdale, Cal., Type B
4. Spiders from bush bean plants..... Berkeley, Cal., Type B
5. Bush beans, some of which were slightly scarred, picked over, washed and packed in clean jars for canning Berkeley, Cal., Type A
6. Manure from large hog which had recovered from botulism 3 months before sample was taken .. . Hollister, Cal., Type B
7. Discolored moldy hay from an outdoor stack.. Oakdale, Cal., Type A

Four cultures were found in which there was evidence of toxin but it was so weak that the toxin-antitoxin tests were not considered reliable. The material from which these doubtful cultures were made is as follows:

1. Earth from spider tube, spider droppings and web.... Berkeley, Cal.
2. Sow bug from bush bean plant Berkeley, Cal.
3. Linnet claws..... Madera, Cal.
4. Spider and small bugs from bush beans..... Oakdale, Cal.

C. This survey is obviously brief and must serve chiefly as an outline for further research. Nevertheless, the following points seem to be quite clearly indicated.

1. *B. botulinus* is widely distributed in nature.
2. *B. botulinus* is present in the garden and may be on the fruit or vegetables when they are picked.

3. *B. botulinus* is not necessarily associated with active decay. It may be present in the blemishes or spots on the skin of apparently sound fruit and vegetables.

D. The strain of *B. botulinus* found in the hog manure of the Hollister series apparently indicates that *B. botulinus* may remain in the intestinal tract of an animal for at least four months after contaminated food has been eaten.

E. *B. botulinus* may not occur far from the habitation of man. Of the five localities visited, only one failed to give positive results as to the presence of *B. botulinus*. This locality, Madera, differed from the other four in being isolated and deserted. There were no human beings living on the place, no domestic animals other than horses, and there was no vegetable garden.

F. The evidence very strongly suggests that *B. botulinus* may be closely associated with or disseminated by spiders or insects common in gardens in California. Since *B. botulinus* grows readily at temperatures as low as 22°C., there is no reason for assuming that this organism must be associated with the stools of warm blooded animals.

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NOTES ON BACILLUS BOTULINUS

G. S. BURKE

From the Laboratory of Bacteriology and Experimental Pathology, Stanford University, California¹

Received for publication, April 5, 1919

The production of heterologous toxins by different strains of *B. botulinus* was first recognized by Leuchs, in 1910. He produced antitoxins in horses for both the Ellezelles and Darmstadt strains and found that the antitoxin of the Ellezelles strain would protect against the toxin of its own strain but not against the toxin of the Darmstadt strain, and vice versa.

In 1917 Dr. E. C. Dickson of the Stanford Medical School, San Francisco, produced antitoxins in three goats, which were immunized against his strains III, IV and VI, respectively. He found that strains III and IV, which he had isolated from outbreaks of botulism in California and Oregon were homologous. Strain VI, however, which was isolated in Albany, New York, from cheese which had caused the death of several persons, produced a toxin which was not neutralized by the antitoxin of either strain III or strain IV. Neither would the antitoxin of strain VI neutralize the toxins of strains III or IV.

By means of toxin-antitoxin tests I have identified twelve strains of *B. botulinus* from material obtained in different parts of California and one from an outbreak of food poisoning in Seattle, Washington. Five of the California strains came directly from outbreaks of poisoning from home-canned vegetables and fruits. One came from hay suspected in a case of forage poisoning in horses. The other six had no direct connection with cases of poisoning. (Burke, 1919 b). These twelve strains fall into two distinct types as shown by the toxin-antitoxin

¹ Aided by a grant from the State Council of Defense of California.

experiments. The antitoxin of one type neutralizes the toxin of its own type but not of the heterologous type. I have designated the two as *B. botulinus* Type A and *B. botulinus* type B. The type A strains usually produce a stronger toxin in broth than the type B strains—but aside from this their cultural characteristics on 2 per cent glucose beef infusion agar, double strength beef infusion broth (2 per cent glucose), brain and cooked meat media are identical. There is no difference in morphology.

Dr. Dickson's strains III and IV, to which reference has been made above, are type A strains. The strain which was isolated from cheese by Dr. Nevins in Albany, New York, and which Dr. Dickson refers to as strain VI in his series, is a type B strain. In September 1918 Dr. Robert Graham of the University of Illinois sent me eight cultures containing *B. botulinus* which he had isolated from material from forage poisoning cases among horses and mules in Illinois and Kentucky and from a human case of botulism, traced to canned beans, in Decatur, Illinois. I have tested these cultures against the two types of antitoxin and have found that Dr. Graham's strains are all homologous with my botulinus type B.

It is a point of interest that all of the above nine strains which were isolated in Illinois, Kentucky and New York State should belong to type B, whereas in the Pacific Coast States Dr. Dickson and I have found type A to be predominant. Out of a total of fourteen strains from eight different localities we have isolated nine type A strains and only five type B strains.

PRODUCTION OF BOTULINUS ANTITOXIN FOR LABORATORY PURPOSES

Goats are the best animals to use in producing antitoxin for laboratory tests. As they are very resistant to the toxin of *B. botulinus*, the dosage can be rapidly increased and a good antitoxin produced within a period of two months.

Where goats are not available a weak antitoxin may be produced in rabbits. This can be satisfactorily used in identifying

strains of *B. botulinus* if the toxin is first carefully titrated to determine the minimal lethal dose.² I succeeded in carrying two rabbits, one for type A and one for type B toxin, through the eighth injection. The initial dose was about one-fourth of a minimum lethal dose. The dose was increased very gradually each week. At the eighth week each rabbit received 10 minimum lethal doses. After this injection one of the animals developed severe symptoms of botulism and it was thought unwise to carry the injections further as five rabbits had already been lost. It was found that the serum of each rabbit contained a weak anti-toxin which would neutralize from six to ten minimum lethal doses of the homologous toxin.

TECHNIQUE FOR ISOLATING *B. BOTULINUS*

B. botulinus may be readily isolated from agar transplants made direct from the contents of cans of spoiled food because there are, as a rule, few other bacteria present.³ When however, materials such as manure and intestinal contents are to be tested, it is difficult to recover the organism from the original agar transplants because material of this sort is rich in strict anaerobes and facultative anaerobes and the number of botulinus bacilli is comparatively small.

The most reliable technique is to inoculate from the original material into cultures of Van Ermengem's broth⁴ and chopped meat media.⁵

² One minimum lethal dose of botulinus toxin is that amount which will kill a 250 gram guinea-pig in forty-eight hours.

³ In one case I have isolated *B. botulinus* from a jar of beans which showed signs of spoilage but in which no toxin could be demonstrated by animal inoculation. The strain was a typical type B strain, producing a moderately strong toxin in Van Ermengem broth culture. This demonstrates the importance of making cultural as well as toxin tests on canned food sent in to the laboratory for examination.

⁴ 1000 grams of lean beef to the liter, 5 grams NaCl, 10 grams Witte's peptone, 2 per cent glucose. The medium oil stratified in the tubes. (See Van Ermengem, E. 1912.)

⁵ Finely chopped beef heart or lean meat is mixed with two volumes of water and brought slowly to the boil, being stirred constantly. It is neutralized, tubed and autoclaved at 15 pounds pressure for 30 minutes.

The broth and meat cultures are richly inoculated with the suspected material. Duplicate cultures are made, one set being heated to 60°C. for one hour to destroy the non-spore bearing organisms. The cultures are then incubated at 28°C. after which portions are filtered and tested for toxin by injecting 1 cc. of the filtrate subcutaneously into a 250 gram guinea-pig. If the pig dies within four days the filtrate is tested against the antitoxins of *B. botulinus* types A and B to determine the presence of specific botulinus toxin. For this purpose the filtrate is diluted with saline so that 1 cc. will kill a 250 gram guinea-pig in approximately forty-eight hours. Three 250 gram guinea-pigs are selected. The first pig receives 1 cc. of diluted filtrate mixed with antitoxin for *B. botulinus* type A. The second pig receives 1 cc. of the diluted filtrate mixed with antitoxin of *B. botulinus* type B. The third pig is the control and receives 1 cc. of the diluted filtrate but no antitoxin. Pure cultures of *B. botulinus* type B produce usually a little more than one minimal lethal dose of toxin per cubic centimeter after three days growth in meat or Van Ermengem broth medium. Type A strains produce a stronger toxin in the same time. In mixed cultures, however, the toxin production may be much delayed and a negative diagnosis should not be made until the cultures have been incubated for three weeks.

The organism is isolated from the broth or meat culture in which specific toxin is demonstrated. In place of the usual anaerobic plating methods it is the practice in this laboratory to use thinly seeded agar shake cultures. After incubation these cultures are examined and fished by transmitted light on the stage of a dissecting microscope such as is used in Zoological laboratories. A lens magnifying 10-20 diameters is most satisfactory. (See E. C. Dickson and G. S. Burke, 1918.)

The development of this technique was necessary because the hydrogen plating methods used at first were too slow to permit of the examination of large amounts of material. Shake cultures are easily and quickly made and there is little chance for contamination. The magnification of 10 to 20 diameters afforded by the dissecting microscope lens gives a clear and detailed

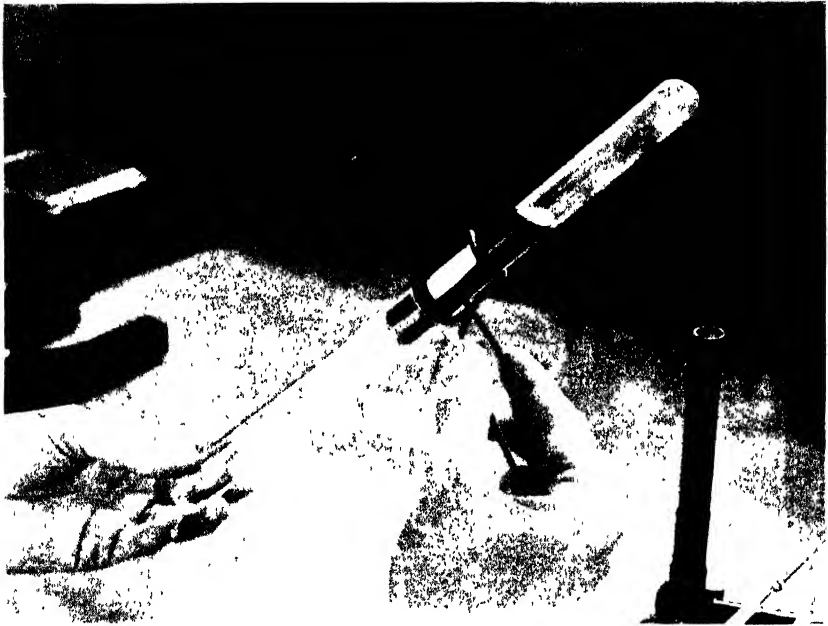
colony picture which is much more satisfactory for the observation of deep agar colonies than that given by the compound microscope. The method has proven so accurate and economical both of time and materials that it seems worth while to give a more detailed description than was given in the former paper.

In detail the technique is as follows. A series of agar shake cultures (5-8) are made from the contaminated Van Ermengem broth cultures, jars of food, etc. (Beef infusion agar, plus 2 per cent glucose, is used, with a reaction as near neutral as possible.) Just before the inoculation is made the agar is boiled for twenty minutes and cooled to approximately 47°C. It is essential to the success of the technique that the agar cultures be thinly seeded in order that the colonies shall be of good size and well separated. Successive dilutions may be used as in plating. As soon as the agar tube is inoculated it is thoroughly shaken and plunged into cold water to harden, after which it is incubated. One very good method of mixing the material in deep agar tubes is to hold the tube in a vertical position grasping it by its upper end. By twisting the wrist the lower end of the tube is then rotated about the vertical axis while the upper end remains practically stationary.

Botulinus colonies may appear in twenty-four hours at 37½°C. or in thirty-six hours at 28°C. Under some circumstances they require a much longer time. (Burke, 1919 a.) In mixed cultures the presence of antagonistic organisms may inhibit the growth. One set of agar cultures should be incubated at 37½°C. and another at 28°C., since *B. botulinus* grows almost equally well at either temperature while some of the contaminating organisms are distinctly inhibited by one or the other temperature. After incubation the colonies in the agar tube are observed and fished under the dissecting microscope by transmitted light.

There are two methods of preparing the agar culture for fishing. In the first, the cotton plug is removed from the culture tube and the tube inverted, with the open end pointed downward into a sterile tube of greater diameter than that of the culture tube. The closed end of the agar culture tube is then passed slowly into the flame of a bunsen burner. The expansion,

caused by the heat, drives the agar column out of the culture tube into the large tube, from which it is emptied into a petri dish for fishing. In the second method a sharp file mark is made around the culture tube about one-half inch from the bottom. The tube is then placed in a large petri dish and broken by applying a piece of red hot glass rod to the file mark. By driving the cotton plug down into the culture tube, the agar



column is pushed out of the broken end so as to expose any desired colony.

The colony chosen for fishing is placed in position under the dissecting microscope lens. The surface of the agar above the colony is sterilized by brushing a red hot platinum loop very lightly over it. The agar should be slightly melted by this operation, giving a smooth glossy surface through which a clear image of the colony can be had. A short platinum needle (1 to $1\frac{1}{4}$ inches) is used for fishing. This is sterilized and plunged down through the sterile agar surface into the colony.

Agar shake cultures are made from the material fished from the colonies. These transplants are as a rule thinly seeded. If they are not pure it is an easy matter to find in them well isolated colonies which can be successfully fished without contamination. Final identification of the isolated organism is made by culturing it in broth or meat and testing the filtrate for specific botulinus toxin.



COLONY CHARACTERISTICS OF *B. BOTULINUS*

Deep agar colonies of *B. botulinus*, as seen under the dissecting microscope with a magnification of 10 to 20 diameters are very characteristic. The description here given is based upon a study of nine pure strains isolated at Stanford University. Seven of the strains were isolated from mixed cultures on the basis of these colony characteristics.

In a young culture the typical *Botulinus* colony (see plates 1 and 2) is a thin translucent disc, having a small opaque knot or "nucleus" on or near the periphery at one side. The edge of the disc is either flattened or sharply indented at the point nearest the knot. The disc is more or less completely filled with clear spots or "vacuoles" which are in reality gas bubbles caught in the meshes of the bacterial growth as shown by examination of stained smears of the colonies. The outline of the colony is always clear cut and definite.

The translucent appearance of the colonies is due to the comparative thinness of the disc and to the presence of the gas bubbles. The gas bubbles may be of any size. Sometimes they are so large that seven or eight of them fill the disc. No photograph was obtained of such a colony. Again they may be so small as to look like little white specks. The gas bubbles when visible are diagnostic of *Bacillus botulinus*. I have been unable to find any other anaerobe producing this type of colony, although I have examined more than 400 cultures from material rich in anaerobes, and also cultures of the common pathogenic anaerobes.

The knot or "nucleus" in a young culture is very small and dense. In old cultures it develops short stubby processes, which are nevertheless translucent. The diameter of the tuft thus formed, however, is never greater than the diameter of the disc.⁶ One of my strains rarely shows any "nucleus" or irregularity of outline, but its gas bubbles are very distinct.

Another very typical feature of *botulinus* colonies is the transparency of the disc in old cultures after three to six weeks incubation. See plate 3, figures 1 and 2.

In plate 2, figure 3, a very small colony can be seen at the edge of the large colony. The disc is comparatively small and

⁶ The pictures of *botulinus* colonies given by V. Hibler (1908) are similar to the colonies of certain anaerobes which I have encountered as contaminants in my work with *B. botulinus*. Colonies of that type with a heavy hairy tuft at one side of the disc are not found in pure cultures of the strains of *B. botulinus* which we have in this laboratory.

has the appearance of a clear gas bubble. This suggests that the opaque knot is the original nucleus of growth and that as gas is produced the agar is split and the bacteria grow into the space thus formed. The agar is sometimes split in more than one plane (plate 1 and plate 3, figs. 4 and 5).

The colony characteristics of *B. botulinus* are the same on either extract or infusion agar and the reaction of the medium does not affect them. The variations which occur are apparently due to a difference in the stiffness of the medium or to the size of the colonies. In very large colonies it is difficult and sometimes impossible to see the gas bubbles.

GENERAL CULTURAL CHARACTERISTICS OF *B. BOTULINUS*

The optimum temperature for *B. botulinus* as originally recorded was 22°–28°C. The pure strains isolated at Stanford, however, grow somewhat more rapidly at 37½°C. than at 28°C. At 37½°C. agar shake cultures show well defined and typical growth in 24 hours. At 28°C. 36–40 hours are required for the same growth, and at lower temperatures the rate of development is still slower. Toxin is produced at 37½°C. as well as at 28°C.

B. botulinus is a proteolytic anaerobe. It digests and darkens but does not blacken meat and brain media. It produces strong toxin in meat cultures and the organisms remain viable for a much longer period in meat and brain cultures than in broth or agar.

In Van Ermengem broth cultures after 40 hours incubation at 28°C. *B. botulinus* produces a delicate clouding of the medium. If the culture is not shaken the broth just under the oil is usually found to be clear. After incubating for a month or more the cloud disappears leaving the broth clear. In a pure culture there is no noticeable sediment in either the young or old cultures. Other observers have described a heavy sediment in old cultures but in my experience a heavy sediment is a sign of contamination.

My strains of *B. botulinus* do not spore readily in either broth

or agar. In brain media spores sometimes appear in two weeks but usually it requires a much longer period.

In infusion agar, 2 per cent glucose, the anaerobe line for *B. botulinus* is one centimeter below the surface. When extract agar is used, however, the anaerobe line drops to two centimeters. For this reason extract agar tubes should contain at least three inches of agar.

SUMMARY

1. There are two known types of *B. botulinus*, which are here designated type A and type B. They produce heterologous toxins and are easily identified by a toxin-antitoxin test.

2. Antitoxin for laboratory purposes may be produced from either goats or rabbits. Goats are the more satisfactory animals.

3. The most reliable method of demonstrating the presence of *B. botulinus* in contaminated material is to make Van Ermen-gem broth or meat cultures, incubate for three weeks, filter and test the filtrate for specific toxin.

4. The use of agar shake cultures, examined and fished under a dissecting microscope, is a simple and reliable technique for the isolation of *B. botulinus* from contaminated material or cultures.

5. *B. botulinus* produces colonies in agar which are characteristic and can be readily identified.

6. The strains of *B. botulinus* isolated at Stanford University grow and produce toxin as readily at 37½°C. as at 28°C. They do not produce a heavy sediment in broth and they do not produce spores readily in either broth or agar.

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PLATE 1

Fig. 1. Deep agar colony of *B. botulinus* from forty-eight hour culture. *d* indicates the edge of a small disc lying in a different plane from the large disc.

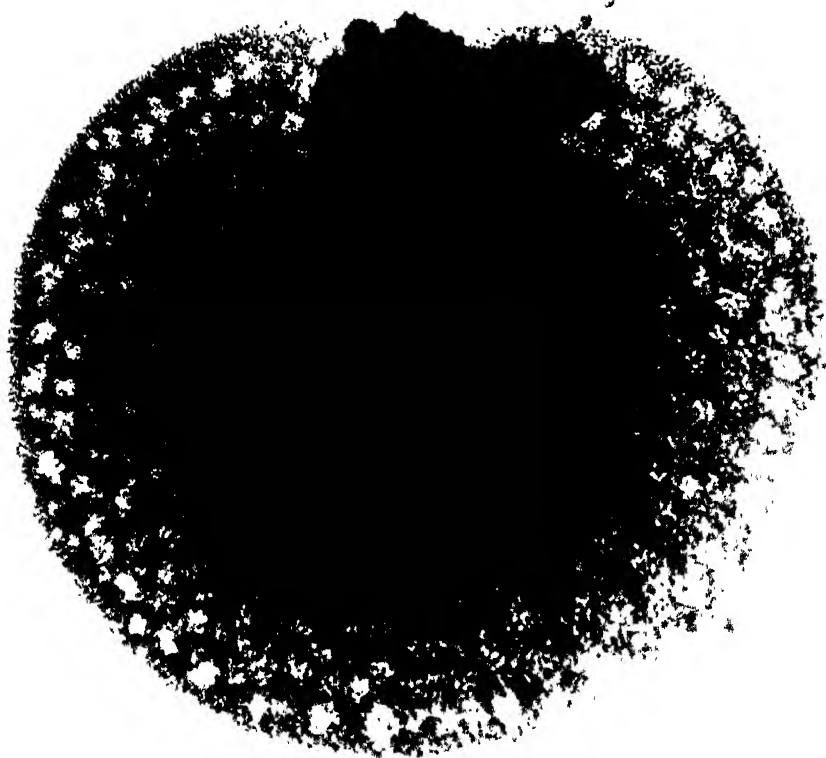
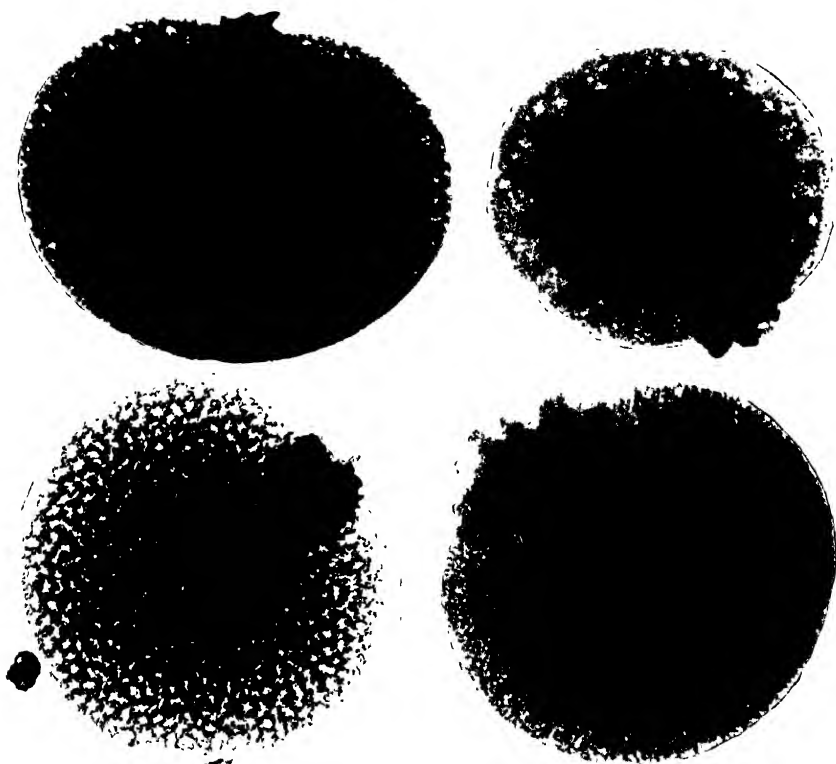


PLATE 2

Figs. 1 and 2. Deep agar colonies of *B. botulinus* from forty-eight hour culture, showing varying size of gas bubbles.

Fig. 3. Deep agar colony of *B. botulinus* from a week old culture, with a very small colony lying near the edge of the large colony.

Fig. 4. Deep agar colony of *B. botulinus* from a week old culture, showing minute gas bubbles in the disc, and branching processes of the "nucleus."



(Burke Notes on *Bacillus botulinus*)

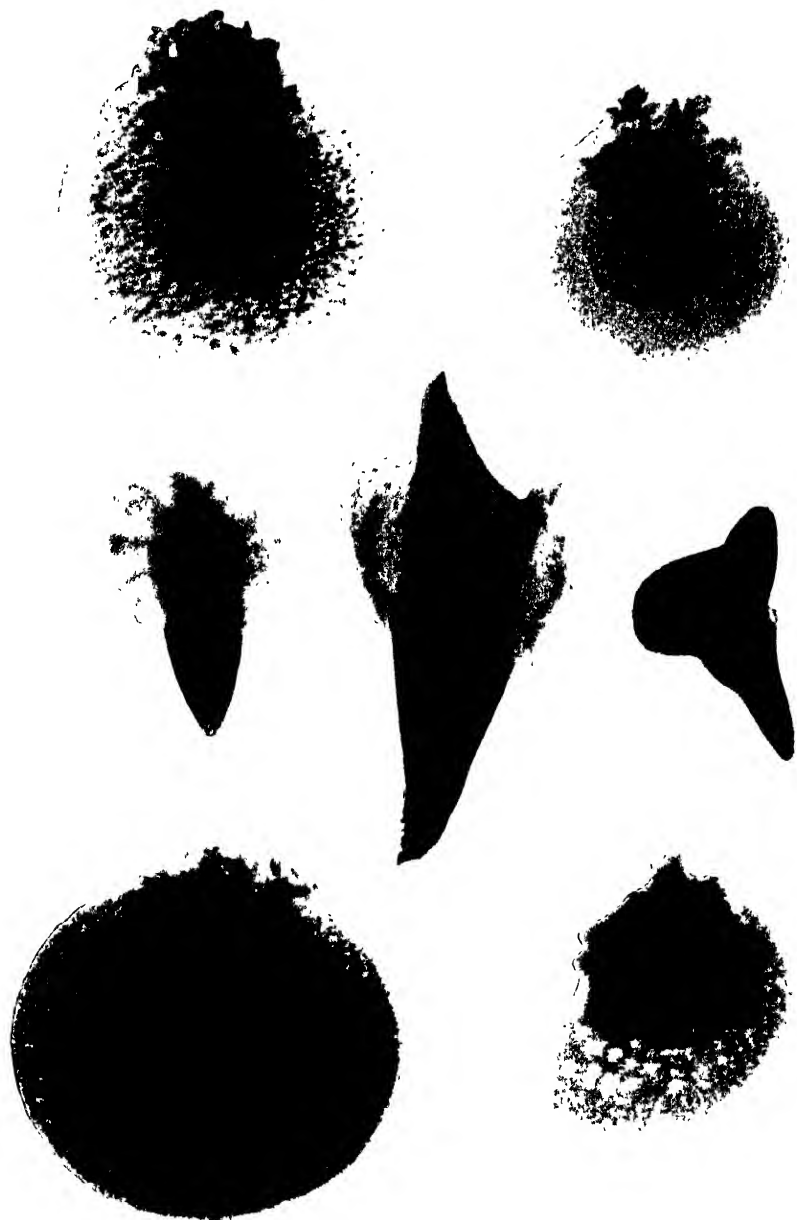
PLATE 3

Figs. 1 and 2 Deep agar colonies of *B. botulinus* from four weeks old culture, showing transparency of disc and branching processes of the "nucleus."

Fig. 3 Deep agar colony of *B. botulinus* from a three weeks old culture. View of the edge of colony.

Figs. 4 and 5 "Composite colonies" of *B. botulinus*, having three discs lying in different planes

Figs. 6 and 7. Two deep agar colonies from the same week old agar culture. Figure 6 shows the minute gas bubbles in the disc and the branching processes of the "nucleus" In figure 7 the growth in the disc of the colony has diffused into the surrounding medium.



(Burke: Notes on *Bacillus botulinus*)

THE SO-CALLED REDUCED OXYGEN TENSION FOR GROWING THE MENINGOCOCCUS

EDWARD F. KOHMAN

From the Laboratory of the Base Hospital, Camp Jackson, South Carolina

Received for publication, April 5, 1919

The following series of experiments was planned to determine the rôle that carbon dioxide plays in the cultivation of the meningococcus. Although they have the limitation of dealing only with two strains, they emphasize the importance of the use of this gas in culturing the meningococcus within certain reactions of the media. The possibility was considered that definite knowledge as to the exact effect of this gas on such cultures might lead to some application of the principle involved to the treatment of meningococcus infections.

Wherry and Oliver (1916) found that thousands of colonies of the gonococcus could be produced by growth at partial oxygen tension when control aerobic tubes remained sterile or showed only a few colonies. In accordance with this Cohen and Markle (1916) made aerobic, partial tension and anaerobic cultures from a case of cerebrospinal meningitis on a medium composed of equal parts of the basic sodium phosphate agar described by Martin (1911), and sterile pleuritic fluid. The pyrogallic acid method was used for the anaerobic cultures, and for the partial tension the culture was connected to a freshly inoculated agar slant of *B. subtilis* by means of a rubber tube. Two colonies appeared on the aerobic tubes, whereas hundreds appeared on the partial tension tubes. The anaerobic cultures remained sterile. In a later article Cohen (1918) again reports favorably on this method and draws the conclusion that the meningococcus is a micro-aerophil, and that the growth ordinarily obtained by aerobic methods consists only of the small minority of meningococci that are resistant to full oxygen tension. Accordingly

Cohen and Fleming (1918) have worked out the optimum amount of air to be replaced by carbon dioxide to culture the meningococcus. Their method has been in use for about six months in the laboratory of the Base Hospital at Camp Jackson. It consists in placing 5 grams of sodium carbonate in a beaker in a 10 liter museum jar containing the cultures either in tubes or on plates. A solution of 5 cc. of sulphuric acid in 35 cc. of water is poured into the beaker and as soon as the violent reaction begins to subside, the cover is clamped down. A tin pail with a close fitting cover has subsequently been used with equally good results. The carbon dioxide thus generated will replace about 10 per cent of the air.

St. John (1919), in a recent paper, concludes that the only advantage in growing the meningococcus in connection with *B. subtilis* and in a partial tension of carbon dioxide comes from the increased moisture in the atmosphere and on the surface of the media under these conditions. He partly replaced the air in Novy jars by means of hydrogen so that the oxygen content varied between 15.6 per cent and 1.2 per cent. He also reduced the oxygen tension by means of *B. subtilis* to amounts varying between 15.3 per cent and 9.5 per cent of atmospheric tension, and reduced the air pressure by means of a vacuum in various degrees. He compared growths obtained under these conditions with that on plates in an atmosphere of air, as well as on plates in a sealed chamber containing moisture. His conclusions are that the moist chamber gives as good or better growth than the other methods. He states that growth is inhibited by carbon dioxide when more than 50 per cent of the air is replaced, but does not say anything as to the effect of the amount recommended by Cohen and Fleming.

It is no doubt true that a moist atmosphere is very beneficial for the growth of the meningococcus, as it probably is in the case of most organisms. But the results of the experiments reported below are in accord with the findings of Cohen and Fleming that the replacement of air by carbon dioxide has a very stimulating effect on the growth of the meningococcus under certain conditions. It did not seem, however, that the

effect could be due to reduced oxygen tension as they claim. The atmosphere contains nearly 21 per cent of oxygen. Replacing 10 per cent of the air with carbon dioxide reduces this figure to between 18 and 19 per cent. The resulting change in pressure is scarcely appreciable as it is within the variations of barometric pressure. But 10 per cent of carbon dioxide in the air is many times the normal figure and carbon dioxide is by no means an inert gas.

In seeking for explanations two other possibilities seemed worthy of consideration in accounting for the effect of carbon dioxide on the growth of meningococcus. It could not be disregarded that the gas might be one of the nutrients necessary for this organism. Such a case would not be without parallel as Nathanson (Loeb, 1917), as well as Winogradsky, have shown. Wherry and Ervin (1918) state that if carbon dioxide is removed from the atmosphere of a culture of the tubercle bacillus, growth is inhibited. Chapin (1918) claims that carbon dioxide has a very beneficial effect on the growth of the gonococcus other than its influence on the reaction of the media in as much as it stimulates growth even in acid reaction. But he does not give the hydrogen ion concentration of the acid reaction used by him. On the other hand the effect of this gas on the reaction of the media seemed the most plausible explanation. The importance of this phase of nutrient media is just beginning to be realized. Cohen and Clark (1918) have recently shown that even in the case of the more common bacteria, *B. bulgaricus*, *B. coli*, *B. aerogenes*, *B. proteus*, *B. dysenteriae*, (Flexner and Shiga), the position of the optimal zone varies enough to prohibit the use of a common reaction.

Let us consider what happens in a slightly alkaline media under a given pressure of carbon dioxide. The gas will be absorbed until the free alkali is converted into sodium bicarbonate. After this reaction has gone to completion the gas will still be absorbed to form carbonic acid, the final concentration depending upon the pressure. A buffer system thus results like that which is so effective in maintaining the constancy of the reaction of the blood stream and other biological fluids, where

the carbon dioxide tension is maintained by the respiratory system. Considerable variation in the pressure does not materially affect the reaction, because appreciable variation in the ratio, $\text{H}_2\text{CO}_3 : \text{NaHCO}_3$, produces but little change in the hydrogen ion concentration. The result then, of an atmosphere containing a certain tension of carbon dioxide, on media of varying degrees of alkalinity is first to reduce all to the same reaction, and then maintain it by a very delicately adjusted buffer system; for as soon as acid substances are produced by the growing organism, an equivalent amount of carbon dioxide is evolved and the reaction remains unchanged.

In the following experiments 2.5 per cent slant agar tubes made with meat infusion and containing 1 per cent Bacto-peptone and 4 per cent defibrinated human blood were used. The hydrogen ion concentration was determined by comparison with standard solutions of known hydrogen ion concentration. For the titration a "comparator" block was used to hold the tubes as represented in the following diagram.

	<i>Media</i>	<i>Water</i>	<i>Media</i>
Second row.	4	5	6
	<i>Standard</i>	<i>Media</i>	<i>Standard</i>
First row.	1	2	3

Into the tubes numbered 2, 4, and 6 was put 5 cc. of the media and this was diluted to 15 cc. Into the tubes 1 and 3 was put 5 cc. of the standard solution, also diluted to 15 cc. Tube 5 contained distilled water. At 1 was placed the standard solution with which the media were to be compared. For 3 one tube was prepared of the standard above and one of the standard below that in 1. These two were interchanged as a guide to get a closer comparison between the media and the standard in 1. Into tubes 1, 2 and 3 were put equal amounts of the particular indicator best adapted to the range desired as recommended by Clark and Lubs (1917). Other variable contents of the media will be noted in the different experiments as they are described. For incubation all tubes were placed in 10 liter museum jars which had moisture in the bottom as well as a tumbler containing water. Vaseline was used to make the

covers fit closely on these jars but no attempt was made to clamp them down to maintain a pressure. To produce an atmosphere containing approximately 10 per cent carbon dioxide, 5 grams of sodium carbonate were placed in a tumbler in the jar and a solution of 5 cc. of sulphuric acid in 35 cc. of water poured over it, the cover being placed on the jar as soon as the reaction began to subside. This will be designated the CO₂-jar. By air-jar is meant a similar jar with similar moisture conditions but without the carbon dioxide.

A strain of meningococcus was first used which was obtained from the blood culture of a case of meningococcus septicemia. When this culture was twenty-four hours old it was transplanted to brain media (ground beef brain with enough meat infusion broth added to cover the brain after being autoclaved) and incubated without carbon dioxide. By propagating the organism in this medium throughout the experiments, a uniform inoculating material was used and the organism did not become accustomed to an atmosphere of carbon dioxide as the experiments progressed. To inoculate the tubes, 0.2 cc. of the supernatant liquid of a brain media culture was emulsified with 5 cc. of broth and into each tube was introduced a uniform drop of this emulsion. This drop, with the water of condensation in the butt of the agar slant, gave an abundance of liquid to flood the entire slant by properly tipping it, which resulted in a uniform seeding and gave a uniformly moist medium. A system of + signs is used to designate the degree or heaviness of growth. One + means the lightest growth obtained in that particular experiment in which it occurs. Five + is the heaviest growth recorded and in that case the individual colonies were 0.4 cm. in diameter. Two + is a satisfactory growth whereas three + and four + both represent a heavy growth.

Experiment I. February 1, 1919. When the first brain media culture was twenty-four hours old it was used to inoculate six blood agar slants, containing 1 per cent glucose, of each of the given reactions. Three of the tubes of each reaction were incubated in the air-jar and the other three in the CO₂-jar. The results after eighteen hours are given in table 1.

TABLE 1

	REACTION OF MEDIA					
	pH = 7.6	pH = 7.8	pH = 8.0	pH = 8.2	pH = 8.4	pH = 8.6
Growth in air-jar.	0	0	0	0	0	0
Growth in CO ₂ -jar.....	+++	+++	+++	++++	+++	+

This experiment was repeated the next day when the inoculating material was forty-eight hours old with the same results.

Experiment II. February 3, 1919. This experiment is a repetition of experiment I with the exceptions that the inoculating culture was seventy-two hours old, and that all the slants, six for each reaction, were set up in the CO₂-jar with 10 per cent carbon dioxide a day previous to inoculation. After inoculation three tubes of each reaction were incubated in the CO₂-jar and three in the air-jar. The results after twenty-hours are tabulated in table 2.

TABLE 2

	REACTION OF MEDIA					
	pH = 7.6	pH = 7.8	pH = 8.0	pH = 8.2	pH = 8.4	pH = 8.6
Growth in air-jar	++	+	0	0	0	0
Growth in CO ₂ -jar....	+++	+++	+++	+++	+++	++

*Only one of the three tubes showed any growth.

In these two experiments the media were the same. It will be noticed that the only conditions under which growth was obtained in an atmosphere of air was for the reaction pH = 7.6 and 7.8 when these tubes had first been kept in a CO₂-jar for a day previous to inoculation. But the growth with these reactions in the air-jar was distinctly inferior to that in similar tubes incubated in the CO₂-jar. Evidently enough carbon dioxide was absorbed the day previous to inoculation to induce some growth by partially adjusting the reaction. This experiment was repeated twice later and in both cases similar results were obtained. From the laws of the solubility of gases it follows that as soon as the tubes are removed from the CO₂-jar they begin to lose the gas absorbed by the media in excess of that which combines with the free alkali, and thus the buffer effect of the carbon dioxide is in part lost. This explains why a less

favorable growth resulted in air even though the media had been in the CO₂-jar on the day previous to inoculation.

Experiment III. February 7, 1919. Six blood agar slants with a reaction of pH=8.2 and containing 1 per cent glucose, and six of the same lot of media but lacking the glucose were inoculated with a twenty-four hour brain media culture. Three of the glucose and three of the glucose-free tubes were incubated in the air-jar and three in the CO₂-jar. After twenty hours incubation all the tubes in the CO₂-jar had a heavy growth of meningococcus, no difference being noticeable between the glucose and the glucose-free media. In the air-jar no growth was obtained and none developed upon twenty-four hours subsequent incubation in the CO₂-jar. Evidently the carbohydrate cannot supply the carbon dioxide or act as a substitute for it under the given conditions, an assumption that might be made if the gas were being used in the metabolism of this organism.

Experiment IV. February 11, 1919. An emulsion of freshly precipitated and washed calcium carbonate was made in the inoculating material, and twelve glucose tubes with a reaction of pH = 8.2 were then inoculated with it. Six of these were incubated in the air-jar and six in the CO₂-jar. At the end of twenty hours the tubes in the CO₂-jar all had a very satisfactory growth of meningococcus whereas the tubes in the air-jar remained sterile. Apparently the insoluble calcium carbonate cannot bring about the satisfactory condition that is produced by the carbon dioxide. This is what would be expected if it is a matter of adjusting the initial reaction.

Experiment V. February 11, 1919. Twelve glucose blood agar slants with a reaction of pH = 8.2 were inoculated in the usual manner. Six of these were incubated in the CO₂-jar and six in a similar jar in which 10 per cent of the atmosphere was replaced by nitrogen gas. This was generated from sodium nitrite and ammonium chloride, washed by passage through a solution of sulphuric acid and one of sodium hydroxide and finally by shaking with a solution of sodium hydroxide. After twenty hours incubation the tubes in the CO₂-jar had a good growth while the tubes in the N₂-jar showed no growth. This is evidence that the carbon dioxide does not produce its effect by diminishing the oxygen tension, with the reaction of media here used. In an experiment given below it will be shown that with more acid reactions, i.e., the optimum for air growths, this gas has an inhibiting effect.

Experiment VI. February 13, 1919. An attempt was made to incorporate the carbon dioxide in the media by means of sodium bicarbonate. In each of six bottles was put 100 cc. of glucose-free agar with a reaction of pH = 7.6. After sterilization 2 cc., 4 cc., 6 cc., 8 cc., and 10 cc. of twice normal sodium bicarbonate was added respectively to five of these bottles, the sixth being used for a control. The addition of 2 cc. of twice normal sodium bicarbonate to 100 cc. of this agar gave it a reaction of pH = 7.9. The reaction of the other lots was not determined. Six tubes of each of these lots of media, with human blood, were inoculated in the given way, three incubated in the air-jar and three in the CO₂-jar. After twenty hours all the tubes in the CO₂-jar had a luxuriant growth whereas in the air-jar only the control tubes, with no sodium bicarbonate, showed any growth and this was scanty. The blood in the tubes containing 6 cc. or more of sodium bicarbonate per 100 cc. of media became very much decolorized upon incubation in the air-jar but retained its bright color in the CO₂-jar where the high alkalinity was neutralized by the carbon dioxide. This fact, together with the results of the growth, is evidence that the organism could not tolerate the more alkaline media produced by the addition of the sodium bicarbonate unless this was neutralized by the carbon dioxide. The experiment was repeated the next day and similar results obtained.

Experiment VII. February 19, 1919. Upon seeing the results of experiment VI, it was decided to make a series of lots of media with a very wide range of reaction. Eight different reactions were made from a lot of agar with a reaction of pH = 7.2 as represented in table 3.

TABLE 3

AMOUNT PER BOTTLE	N/1 HCl ADDED	pH	N/1 NaOH ADDED
cc.	cc.		cc.
250	6.0	4.7	0
250	4.0	5.3	0
250	2.25	6.0	0
250	1.5	6.7	0
250	0	7.2	0
250	0	7.8	1.0
250	0	8.4	4.5
250	0	9.0	8.0

For the two most alkaline reactions there was considerable flocculation during sterilization and a resultant decrease in alkalinity. Consequently a portion of the normal solution of sodium hydroxide was

added after sterilization to bring back the desired reaction. Six tubes of each reaction were inoculated with a four day old brain medium culture of the meningococcus. Three were incubated in the air-jar and three in the CO₂-jar. The results are tabulated in table 4.

TABLE 4

	REACTION OF MEDIA							
	pH = 4.7	pH = 5.3	pH = 6.0	pH = 6.7	pH = 7.2	pH = 7.8	pH = 8.4	pH = 9.0
Growth in air-jar, 24 hours . .	0	0	0	0	0	0	0	0
Growth in CO ₂ -jar, 24 hours	0	0	0	0	++	++	++	++
Growth in air-jar, 48 hours .	0	0	0	0	0	0	0	0
Growth in CO ₂ -jar, 48 hours	0	0	0	++	++++	++++	++++	++++

This experiment was repeated the next day, using the same culture for inoculation, which was then five days old, and similar results were obtained.

Experiment VIII. February 21, 1919. In this experiment six tubes of each of the reactions used in the previous experiment were inoculated with a twenty-four hour brain medium culture which was made from the five day old brain medium culture used in the previous experiment. Three tubes of each reaction were incubated in the air-jar and three in the CO₂-jar. The results are tabulated in table 5.

TABLE 5

	REACTION OF MEDIA							
	pH = 4.7	pH = 5.3	pH = 6.0	pH = 6.7	pH = 7.2	pH = 7.8	pH = 8.4	pH = 9.0
Growth in air-jar, 24 hours	0	0	0	+++	++	+	0	0
Growth in CO ₂ -jar, 24 hours	0	0	0	+	++++	+++++	+++++	+++++

It will be noticed that the growth in the air-jar for the reaction pH = 6.7 was decidedly better than that for the same reaction in the CO₂-jar; but for the reaction pH = 7.2 the reverse is true, and for the reaction pH = 7.8 the growth is much heavier in the CO₂-jar than in the air-jar. The results of this experiment, together with the previous ones, may be interpreted as follows. This organism is very susceptible

to the reaction of the medium. The optimum reaction is found in a medium in which the pH = 7.0 or thereabout. The tension of the carbon dioxide used tends to bring all reactions more alkaline than this down to this figure. As the organism grows it produces acids which displace an equivalent amount of carbon dioxide and therefore do not greatly increase the acidity. This also explains a phenomenon noticed throughout these experiments, i.e., the first growth appears at the upper end of the slant, where the medium is thin and therefore effected more quickly by the carbon dioxide; but the final growth is heaviest on the lower end of the slant. In the CO₂-jar the carbon dioxide apparently increases the acidity of an initial reaction of pH = 6.7 enough to exert an inhibiting effect. This serves as additional evidence that this gas exerts its effect entirely by adjusting the reaction of the medium. At any rate any other effect is overshadowed in this experiment. The fact that frequently growths were obtained in the CO₂-jar with the less viable inoculations, as will be shown in the experiments given below, when no growth was obtained in the air-jar with any of the reactions used may find its explanation in the closer adjustment of the reaction of the medium to the optimum by the carbon dioxide than was secured by titration.

Experiment IX. February 23, 1919. Six tubes each of glucose and of glucose-free blood agar, reaction of pH = 8.0, were inoculated in the given manner and half of each lot incubated in the air-jar and half in the CO₂-jar. In this case both jars were thoroughly dried. To replace 10 per cent of the atmosphere in the CO₂-jar one liter of carbon dioxide was run into the jar by means of a rubber tube under a tuft of cotton in the bottom to prevent too rapid diffusion and escape of the gas. The gas was measured by replacement with water. A similar tuft of dry cotton was placed in the bottom of the air-jar. At the end of twenty hours all the tubes in the CO₂-jar, both glucose and glucose-free had a luxuriant growth whereas in the air-jar only one tube showed any growth and it was very scanty. Here again no difference was noticeable in the growth on the glucose and the glucose-free media in the CO₂-jar. This experiment is evidence that the effect of the carbon dioxide is not a moisture effect.

In the last three experiments a number of the tubes were contaminated by a Gram positive bacillus, probably *B. subtilis*. This contamination was only in the butt of the tube and did not obscure the growth of meningococcus in the upper part of

the slant. Moreover, for every different reaction used, at least one of the three tubes was not contaminated. No difference in the density of the growth, or other evidence of symbiotic effect, could be noticed in the contaminated and the uncontaminated tubes of like reaction and otherwise similar conditions.

Experiment X. February 27, 1919. Another attempt was made to incorporate the carbon dioxide in the medium by means of sodium bicarbonate, beginning with a more acid reaction. Agar was made up to a reaction of pH 6.5, and 100 cc. put into each of six bottles. The pH after sterilization was 6.8. One bottle was used as a control, and to the other five was added respectively, just before pouring the slants at a temperature of about 45°C., 0.2, 0.4, 0.8, 1.5, 2 grams of sodium bicarbonate. Even the addition of 0.2 gram gave a slight evolution of gas at this reaction and the alkalinity therefore increased to a pH of about 7.2. Six tubes of each of the different lots of media just described, containing the given amount of defibrinated human blood, were inoculated as before with a three day old brain media culture, and six with a twenty-four hour culture. The results after eighteen hours incubation are given in table 6.

TABLE 6

	GRAMS OF NaHCO ₃ PER 100 CC. MEDIA					
	Control	0.2	0.4	0.8	1.5	2.0
<i>Results from three day old culture</i>						
Growth in air-jar.....	0	0	0	0	0	0
Growth in CO ₂ -jar... ..	0	+	+++	+++	0	0
<i>Results from one day old culture</i>						
Growth in air-jar	+++	0	0	0	0	0
Growth in CO ₂ -jar	+	++++	++++	+++	0	0

The results of this experiment bear out the statement above that vigorous organisms grow in an atmosphere of air within a narrow range of reaction; the acidity of the optimum reaction in air is increased by the carbon dioxide used sufficiently to inhibit the growth; with more alkaline reactions this gas produces a condition which is much more favorable and will give growth with the less viable organisms which will no longer grow under the conditions used without the carbon dioxide.

Experiment XI. March 1, 1919. The various reactions of media described in table 3, in which the contamination occurred, were again inoculated, six tubes of each reaction with a five day old culture, six with a three day old culture, and six with a one day old culture. The results after twenty hours incubation are given in table 7.

TABLE 7

	REACTION OF MEDIA							
	pH = 4.7	pH = 5.3	pH = 6.0	pH = 6.7	pH = 7.2	pH = 7.8	pH = 8.4	pH = 9.0
<i>Results from five day old culture</i>								
Growth in air-jar	0	0	0	0	0	0	0	0
Growth in CO ₂ -jar	0	0	0	0	+	++	+++	+
<i>Results from three day old culture</i>								
Growth in air-jar	0	0	+	+++	++	+	0	0
Growth in CO ₂ -jar	0	0	0	+	++++	++++	+++	0
<i>Results from one day old culture</i>								
Growth in air-jar	0	0	+	+++	++	+	0	0
Growth in CO ₂ -jar	0	0	0	++	+++	++++	++++	+++

It will be noticed that in the above experiment this strain of meningococcus shows an inclination to grow within a wider range of reaction than in earlier experiments. Even the three day old culture gave a very satisfactory growth in air although not as abundant as in the CO₂-jar. The impression that one received from working with this organism was that in the later experiments it gave a more vigorous growth than in the earlier ones. This was probably due to its becoming accustomed to the artificial media. However the growth obtained above with the one day old inoculating material was distinctly more luxuriant than was obtained with the three day old material, a fact that could not be registered with the system of + signs.

When the above experiments were completed another strain of meningococcus was isolated in the laboratory from a case of cerebrospinal meningitis. Several experiments, similar to those described with the varying reactions of the media, were performed with it and this strain showed the same tendency, in as

striking a manner, to give better growth with the alkaline media in the CO₂-jar than was obtained in air with the most favorable reaction.

CONCLUSIONS

Experiments were conducted with two strains of the meningococcus. The optimum reaction for twenty hour growths on 4 per cent defibrinated human blood agar lies between a pH of 6.7 and 7.4 when incubated in an atmosphere of air. Much better growths can be obtained by making the media with a pH lying between 7.6 and 8.4 and incubating in an atmosphere in which 10 per cent of the air is displaced by carbon dioxide. Frequently the less viable inoculations failed to grow in an atmosphere of air on any reaction of the media used, while a good growth was obtained if 10 per cent of the air in the atmosphere was replaced by carbon dioxide. This was explained by the closer adjustment of the reaction of the media to the optimum by means of the carbon dioxide and by the buffer effect of the equilibrium set up between the gaseous carbon dioxide and the carbonic acid and sodium bicarbonate in the medium. As the organism grows it produces acids which displace an equivalent amount of dissolved carbon dioxide and therefore the acidity is not greatly increased. It is possible that the principles here involved may be applied equally well to the growing of other delicate organisms. By making media with a pH of 7.8 to 8.0 and incubating the meningococcus in a partial atmosphere of carbon dioxide the same media may be used for this organism which are used for the pneumococcus and the streptococcus.¹

¹ Since the above article was written a paper has appeared by Doctor Gates in the Journal of Experimental Medicine for April vol. xxix, Pp. 325, in which Doctor Gates has similarly shown that the effect of carbon dioxide on the growth of meningococcus is really due to its effect on the reaction on the medium. His experiments do not, however, bring out the fact that at times a more vigorous growth is obtained if the alkalinity of the medium is reduced to the optimum by carbon dioxide rather than by a mineral acid. Theoretically this is no more than would be expected, since there is an equilibrium between the carbon dioxide in the atmosphere and that in the solution.

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THE NOMENCLATURE OF THE ACTINOMYCETACEAE

R. S. BREED AND H. J. CONN

New York Agricultural Experiment Station, Geneva, New York

Received for publication, April 11, 1919

In a paper, recently published but written early in 1917 (Breed, Conn and Baker, 1918), the authors criticised some of the ideas underlying the report presented by the Committee on Characterization and Classification appointed by the Society of American Bacteriologists (1917). These criticisms were presented verbally at the 1917 meeting of the Society and as a result one of the authors (Breed) was asked to join the Committee in its work. Soon afterward the task was assigned to him of determining the type species for the genera *Pseudomonas*, *Actinomyces* and *Nocardia*. As the authors believe that such types ought not to be selected arbitrarily, a search of the literature has been made which has brought out many facts of interest. Certain of the conclusions reached are presented in the following paper, which will be followed by another on the nomenclature of the *Pseudomonadaceae*.

GENERIC TERMS USED IN THE ACTINOMYCETACEAE.

The Committee referred to has recommended that two genera be recognized in this family; *Actinomyces*, with *A. bovis* Harz as the type species, and *Nocardia*, with no type species named. The former genus is intended to include the anaerobic pathogens while the second is to include aerobic species, primarily saprophytic in nature.

Every student of the group realizes that the status of these terms has never been well established and that there is great diversity in usage. The confusion which exists has made it very difficult to get at the facts and has necessitated the study

of a large number of papers some of which have proved to be rare and not obtainable in any American libraries. While the references in the following pages have been made with care, it is scarcely possible that all mistakes have been avoided, or that all significant articles have been found. The authors would therefore greatly appreciate the correction of any mistakes which others may discover.

The first organism of this family which was described and named was *Streptothrix foersteri* Cohn, 1875, p. 186. It is a parasitic species occurring in concretions found in the human tear duct. While Cohn's figure of this species and his original description make it clear that his organism was one showing true branching of its filaments, yet Cohn himself did not feel entirely sure of the matter as is shown by the fact that on p. 204 he includes *Streptothrix* (followed by a question mark) with *Cladothrix* under "Zellfaden durch falsche Astbildung verzweigt." The type of branching found in *Cladothrix* was stated correctly by Cohn as false, but was confused by Cienkowski (1877) who reports that at times the branches of *Cladothrix* seem to arise by true, not false, branching.

In view of this uncertainty it is not surprising to find that Winter (1879) actually included *Streptothrix foersteri* under the genus *Cladothrix*, stating in his characterization of the genus that all species in the genus show false branching. This unfortunate mistake appears again and again in the literature of the following decade and was greatly furthered by the confusing mistakes which Macé made. This author in 1888 described a true streptothrix under the name *Cladothrix dichotoma*. Then he later copied Cohn's figure of *Streptothrix foersteri* into the first edition (1889) of his text book under the caption *Cladothrix dichotoma*. In the second edition (1891), this error was continued by substituting a drawing of his own of a true streptothrix still under the caption *Cladothrix dichotoma*. In the third edition of his book (1897) he continues to use the generic term *Cladothrix* in place of *Streptothrix* Cohn and defends this usage on the ground that Cohn was not justified in making two genera for the species which he described under the names

Streptothrix foersteri and *Cladothrix dichotoma*, and that since *Streptothrix* was preempted by Corda, *Cladothrix* remains as the earliest valid name for the genus.

Other authors and especially those of recent years are however practically all in agreement that the filamentous thread bacteria with false branching which occur in fresh water should be placed in the genus *Cladothrix* Cohn or the genus *Sphaerotilus* Kützing (1833) entirely separate from those organisms which have been properly placed in the genus *Streptothrix* Cohn.

Migula's (1895) acceptance of the union of the genera *Cladothrix*, *Streptothrix* and *Actinomyces* in Engler and Prantl's *Die Natürlichen Pflanzenfamilien* under the term *Cladothrix* is later repudiated in his *System der Bakterien* (Migula 1900) where he uses *Sphaerotilus* in place of *Cladothrix* and excludes the species included under *Streptothrix* as belonging to the true fungi. However, Engler (1907), apparently without realizing this, accepts Migula's earlier conclusion and even makes the original mistake worse by using the combination *Sphaerotilus bovis* (Harz) Engler and implying that all other Actinomycetaceae should be placed in *Sphaerotilus*.

The second organism of this family which was found and described was the organism causing lumpy jaw (actinomycosis) of cattle. This was described in 1877 by Harz at Bollinger's request in a paper read before a scientific society in Munich. In publication however the name given by Harz to this organism (*Actinomyces bovis*) appears first in a paper written by Bollinger (1877, p. 485) where credit is given to Harz for having suggested the name. The following year, before the publication of the description of the species by Harz, Rivolta (1878) used the name *A. bovis* Harz in the title of his paper; but later in the body of his paper (p. 208) changed the name for trivial reasons to *Discomyces bovis*. The description written by Harz appeared in 1879 at which time Harz took exception in an appendix (p. 140) to the change of name made by Rivolta thereby showing clearly that Rivolta's paper was already in print. Later Rivolta (1884) announced himself as satisfied to retain the name *Actinomyces* proposed by Harz, both he, and also Harz, being ignorant at the

time that the generic term *Actinomyce* had already been used by Meyen (1827) for one of the higher fungi.

The confusion was increased by Afanasiev (1888), who claimed that *A. bovis* should be united with the genus *Cladothrix* as then known. Nevertheless in a footnote given on p. 84 of the German edition of this paper he remarks in a casual manner that one should use the name *Bacterium actinocladothrix* for this species, and this combination also appears in a German review of this article by Dittrich (1888). In a later paper read by Afanasiev and Schultz (1889) before the Third Congress of Russian Physicians in St. Petersburg, the original of which was printed in Russian, these authors use the term *Actinocladothrix* as if it were a generic name of equal rank with *Cladothrix*; and in at least two of the German reviews of this paper (Afanasiev and Schultz, 1889a and 1889b) *Actinocladothrix* is likewise used as if it were the name of a genus. It is therefore not surprising to find that Gasperini (1892, p. 183) and several later authors attribute the combination *Actinocladothrix bovis* to Afanasiev or to Afanasiev and Schultz. Apparently however this combination was never actually used by them.

Almost at the same time, Trevisan (1889) recognized that *Streptothrix foersteri* Cohn and *Actinomyces bovis* Harz were closely related and he placed both in a new genus *Nocardia*, distinct from *Cladothrix*. Unfortunately in doing this he continued Winter's mistake and described both *Nocardia* and *Cladothrix* as showing false branching. The new generic term was given on the ground that *Streptothrix* had been preempted by Corda (1839, p. 27) for another genus of fungi and that *Actinomyce* had been used by Meyen (1827). While Trevisan knew of the term *Discomyces* Rivolta as shown by the fact that he quotes *D. bovis* in his list of synonyms, he ignores its claim for recognition. In the same list of synonyms he credits the combination *Bacterium actinocladothrix* to Afanasiev. The new name, *Nocardia*, is given in honor of Nocard and it is evident from the fact that Trevisan places the species described by Nocard (1888) as the "bacille du farcin" as the first species in the genus under the name *N. farcinica* that he wished to make this species the type of the genus.

Sauvageau and Radais (1892) placed *Streptothrix foersteri* Cohn, *Actinomyces bovis* Harz, *Nocardia farcinica* Trevisan and other related species in the genus *Oospora* Wallroth (1833, p. 182) claiming that they were similar to the species already included in this genus. Likewise Thaxter (1891, p. 159) in describing the organism causing potato scab (now known to be very similar to the three species named above and usually placed in the same genus with them) named it *Oospora scabies*. However, further investigations have shown this combination of genera suggested by Sauvageau and Radais to be an unnatural one and it has not been generally accepted.

The status of the generic term, *Carterii*, proposed by Musgrave, Clegg and Polk (1908, p. 470) is sufficiently explained for all systematists by merely quoting their statement. After accepting *Streptothrix* as the term which they use for the pathogenic actinomycetes studied by them, they add the following:

In making this decision, we are fully aware of the rights of those who favor *Actinomyces* or *Nocardia*, and under the circumstances are tempted to introduce a new name (*Carterii*) for the genus, together with a full and complete description.

A name proposed in this way is merely a nuisance to all who wish to see biological nomenclature placed on a stable footing.

Thus eight different generic terms (*Streptothrix*, *Cladothrix*, *Sphaerotilus*, *Actinomyces*, *Discomyces*, *Actinocladothrix*, *Nocardia* and *Oospora*) have been more or less generally used for the group of species under discussion; and confusion has increased as the years have passed because increasing knowledge has shown that the species on which these genera were founded were inadequately described by their authors.

Reference to Cohn's original description of *S. foersteri* shows that the only possible way in which this species can be recognized is in case it is shown that its habitat (concretions in the human tear duct) is sufficiently characteristic to identify it. The drawings given and the descriptions of morphology would apply equally well to any other species in this genus.

The situation is even worse in regard to *A. bovis* Harz for a dispute exists in regard to the nature of this organism. Some following the lead of Boström (1891) contend that it is an aerobic organism. Others have even regarded it as similar to, if not identical with certain common aerobic soil forms. On the other hand, Wolff and Isreal (1891) and later Wright (1905) describe the organism causing actinomycosis as an anaerobe, and Wright goes so far as to regard it as sufficiently distinct from the soil forms to place it in a genus separate from them. His view is nevertheless disputed recently by Pinoy (1913) who contends that there are two types of actinomycosis caused by different organisms and that the specific name "*bovis*" should be retained for the aerobic organism.

A very similar situation exists in regard to the organism causing potato scab (*Oospora scabies* Thaxter). That this organism belongs to the Actinomycetaceae is well established today; but Lutman and Cunningham (1914) have recently claimed, without reporting any inoculation experiments, that it is identical with *Actinomyces chromogenus* Gasperini, a common soil form. Krainsky (1914) has shown however that by the use of proper synthetic media, this "species" of Gasperini's may be separated into several distinct types; and the more recent work of Waksman and Curtis (1916), Drechsler (1919) and of one of us (Conn, 1917) has revealed the existence of many more distinct species. While all of the soil forms appear to be more closely related to the potato scab organism than to the forms causing actinomycosis and farcy, only a few of them agree with the potato scab organism even in cultural characteristics, and for these few, conclusive proof that they can cause potato scab (i.e., inoculation experiments) is still lacking.

When the cultural studies made by Krainsky, Waksman and Curtis, and one of us (Conn) are viewed in the light of the morphological studies of these species made by Lachnar-Sandoval (1898), Neukirch (1903) and more recently by Drechsler (1919), it becomes increasingly probable that future investigators will distinguish many species in the group and that they will find them as distinct and as limited in their distribution to specific habitats

as are other species of fungi. While the animal pathogens are better known today than are the soil saphrophytes, and very properly serve as type species for genera, they apparently represent but a small and highly specialized portion of the group of species included in the family Actinomycetaceae.

SUGGESTED SUBDIVISIONS OF THE GENUS ACTINOMYCES

Wright (1905) was apparently the first to suggest the subdivision of the genus Actinomyces Harz. This he does in such a way as to retain the name Actinomyces for the anaerobic organisms which he studied and which he regards as the true *Actinomyces bovis* Harz. However, apparently without having made any special study of the organisms concerned, he accepts the view that the organism causing bovine farcy (*Nocardia farcinica*) is closely similar to the soil saprophytes and suggests that the generic term, Nocardia, be used as an omnibus term to include all species except the anaerobic *A. bovis* and such other anaerobes as may later be identified. This view may be commended for the simple way in which it disposes of all species of no particular interest to pathologists; but it will hardly commend itself to systematists.

Pinoy (1913) disregarding the previous subdivision by Wright also subdivides the genus Actinomyces (for which he uses the term Nocardia). Since he finds that, while the more common type of actinomycosis is caused by an anaerobic organism, a less common type is caused by an aerobic species of the type described by Boström (1891), he regards *Nocardia bovis* (Harz) Blanchard 1896, p. 857, as an aerobic species. On the other hand he places the anaerobic organism described by Wolff and Isreal (1891) and by Wright (1905) in a new genus Cohnistreptothrix (p. 931). As a specific term he uses *isreali*, the specific term proposed by Kruse (1896, p. 56) for the organism described by Wolff and Isreal (1891). Pinoy likewise includes Cohn's original species in this new genus and from the form of the new generic term evidently regards *Cohnistreptothrix foersteri* (Cohn) as the type species of this new genus.

Discussion

Much of this confusion has arisen needlessly and because of ignorance of generally accepted principles of nomenclature. It offers a strong argument in favor of a general study of these principles by pathologists and bacteriologists. American bacteriologists having accepted the International Rules of Botanical Nomenclature (1905 and 1910) as a guide, this code will ordinarily be referred to in the following discussion. Inasmuch however as this code makes no reference to the selection of type species, reference will at times be made to the International Rules of Zoological Nomenclature (1915) in which specific rules governing the selection of type species are given.

Under both of these codes the original and frequently used term *Streptothrix* Cohn must be rejected because of the preemption of the term by Corda (1839) for an entirely different genus of fungi. Corda's term is moreover in common usage among mycologists today (see for example Stevens, 1913, p. 599). Even the most ardent advocate of the establishment of the validity of scientific names through usage will scarcely contend that *Streptothrix* Cohn should be retained when the list of species included in *Streptothrix* Corda is examined¹ and it is realized that new species have been placed in this genus as lately as 1914 and that specimens of these fungi are included in such a widely distributed and well known herbarium as that of the North American Fungi of Ellis and Everhart. The necessity for discarding *Streptothrix* Cohn is doubly emphasized by such an error as that of Stevens (1913, p. 599) who places *S. dassonvillei* Brocq-Rousseau (1907) under *Streptothrix* Corda although

¹ Genus *Streptothrix* Corda, 1839. Corda included a single species, *S. fusca* in the genus. Others have added the following species:

S. abietina Peck (Original description Buffalo Soc. Nat. Hist. Bull. No. 1, p. 69, 1873. Also in 25 Ann. Rept. N. Y. State Mus. Nat. Hist., p. 93, 1873).

S. atra Berkeley and Curtis (Grevillea, 3, 107, 1875).

S. glauca Ellis and Everhart (Jour. Mycol., 4, 107, 1888).

S. cinerea Morgan (Jour. Cincinnati Soc. Nat. Hist., 17, 44, 1895).

S. pereffusa Sumstine (Mycologia, 6, 34, 1914).

the author of this species places it in the very different genus *Streptothrix* Cohn.

The situation in regard to the use of the term *Actinomyce* (not *Actinomyces*) by Meyen (1827) is not quite the same as that just discussed. As early as 1830, Meyen himself points out that his species *A. horkellii* is identical with *Tremella meteorica* Persoon; and so far as the authors of the present paper are aware this is the last appearance of *Actinomyce* Meyen in the literature except as a synonym or homonym. Under these conditions, it is not only possible but necessary under the International Botanical Rules (see Chap. III, Sect. 2, Art. 20 and Chap. III, Sect. 6, Art. 50) to establish the term *Actinomyces* Harz as a *genus conservandum* by action taken at an International Botanical Congress. Pending such action, it is to be hoped that botanists and bacteriologists will continue to use *Actinomyces* Harz. Such usage would be encouraged if action were taken by the Society of American Bacteriologists recommending that *Actinomyces* Harz, not *Actinomyce* Meyen, be recognized as a valid genus.

The only valid argument which can be brought against the use of *Actinomyces* is the one which is brought forward by those who believe in the strict application of the Law of Priority. For such, as ably explained by Blanchard (1900), the term which must be accepted is *Discomyces* Rivolta. Neither the fact that this term has an unfortunate resemblance to the commonly used *Discomycetes* nor the fact that Rivolta (1884, p. 183) himself, in ignorance of the true state of the case, accepted Harz's name in a later publication invalidates *Discomyces*. The fact however that *Discomyces* has fallen into almost complete disuse because of these things gives strong reason for not regarding priority in this case.

No one, so far as known, has attempted to establish the valid status of *Actinocladothrix*, while Blanchard (1900) has explained why *Nocardia* has no standing as a generic term for the entire group of organisms under discussion.

Oospora, *Sphaerotilus* and *Cladothrix* may be dismissed from consideration for, as already indicated, there is general agreement

that the organisms properly included under these terms should not be included in the same genus or even in the same family with the Actinomycetaceae.

Two attempts have already been made to establish the validity of a generic name for this group of organisms through legislative action both of which must be regarded as abortive. The first was taken by a Committee of the English Society of Pathologists who, as reported by Foulerton (1912, p. 304), approved the term *Streptothrix* Cohn in ignorance of the general and wide usage of *Streptothrix* Corda. The second was taken by the Botanical Section of the First International Congress of Comparative Pathology according to Pinoy (1913, p. 933) and confirmed by him in a letter dated September 9, 1918. The name accepted was *Nocardia*. The official record of the Congress however contains no reference to this action which is stated to have been taken during the discussion of a paper by Potron (1912). As this action ignores the stronger claims of *Actinomyces* and *Discomyces*, and does not appear in the official record, it cannot be regarded as final.

Nocardia Trevisan is valid only in case the species *N. farcinica* is placed in a genus distinct from *Actinomyces bovis*. The present justification for this separation turns upon the identity of *A. bovis*. If *A. bovis* is an aerobic species as described by Boström (see p. 590) it is apparently so similar to *N. farcinica* that there is scant justification for placing the two species in separate genera, and *Nocardia* must remain a synonym of *Actinomyces*. If however, the difference between these species is sufficient to justify separating the genera then *Nocardia* becomes a valid term with *N. farcinica* as the type species.

In our judgment neither Wright's nor Pinoy's investigations, based as they are on the study of the pathogens only, justify separating the genus into two parts. Future investigations may well show that there is a closer resemblance between the organism causing bovine farcy and the organism causing actinomycosis than between the farcy organism and soil saphrophytes. Attempts so far made to subdivide the genus appear to us as premature and certain to lead to further confusion in the nomenclature of the group.

TYPE SPECIES FOR THE GENERA STREPTOTHRIX, ACTINOMYCES,
NOCARDIA AND COHNISTREPTOTHRIX

Much of the confusion in the use of these terms would have been avoided if investigators had observed the rules governing the selection of type species generally observed by systematists, and definitely formulated in the International Rules of Zoological Nomenclature, Article 30 (1915).² Many men have regarded the four terms quoted as exact synonyms when, as a matter of fact, since three different species are properly regarded as types of the four genera mentioned, they are exact synonyms only so long as these three species are included in the same genus. A brief discussion of the status of each of the three species with a list of synonyms follows:

Streptothrix, Cohn. In case this term is used, *S. foersteri* Cohn must be recognized as the type species as it was the only species named at the time the genus was proposed (mono-typical genus). This species has often been placed in other genera by writers who consider *Streptothrix* Cohn invalid, and some of these writers wrongly consider the early date at which this species was described to necessitate its acceptance as the type species of any genus which includes it. Thus Vuillemin (1913) incorrectly accepts it as the type species of *Nocardia*.

It is generally recognized that the type species of any genus must have been included in the genus at the time that it was originally described. For this reason *S. foersteri* cannot be regarded as the type in case the terms *Actinomyces*, *Discomyces* or *Actinocladothrix* are used. Trevisan did list *N. foersteri* (syn. *S. foersteri*) as one of the species of *Nocardia* in his original description of the genus; but he plainly indicated (1) by the name used for the genus and (2) by the fact that he placed *N. farcinica* as the first species in the genus that he regarded the latter species as the type of the genus. The right of an author

² After this was written it was discovered that the Committee on Generic Types of the Botanical Society of America have recommended practically the same rules as those used by the Zoologists (see the report of this Committee which has just appeared in *Sci.*, N. S., 49, 333-336. 1919.)

to fix the type of a new genus ought to be universally regarded, and is so regarded by all who observe generally accepted rules of nomenclature.

Synonymy

Streptothrix foersteri Cohn, 1875, p. 186.

Syn. *Cladothrix foersteri* (Cohn) Winter, 1879, p. 60.³

Nocardia foersteri (Cohn) Trevisan, 1889, p. 9.

Oospora foersteri (Cohn) Sauvageau and Radais, 1892, p. 252.

Actinomyces foersteri (Cohn) Gasperini, 1894, p. 684.

Discomyces foersteri (Cohn) Gedoelst, 1902, p. 176.⁴

Cohnistreptothrix foersteri (Cohn) Pinoy, 1913, p. 937.

Actinomyces. For those persons who recognize the validity of *Actinomyces* the matter of the type species is simple, so far as nomenclature is concerned, as *A. bovis* Harz was the only species named at the time the genus was named (monotypical genus). As already indicated however the identity of this species is far from being satisfactorily established.

If any are inclined to recognize the validity of *Discomyces* or *Actinocladothrix*, the same species must serve as type and for the same reason.

Synonymy

Actinomyces bovis Harz (see Bollinger, 1877, p. 485).⁵

Syn. *Discomyces bovis* (Harz) Rivolta, 1878, p. 208.⁶

Bacterium actinocladothrix Afanasiev, 1888, p. 84.⁷

³ The date given (1879) is the date when the manuscript was completed. The completed volume in which this combination appears was not published until 1884, but from the fact that Zopf (1882, p. 13) refers to Winter it is evident that the portion of the volume in which *Cladothrix* is described, was published earlier than 1882. Because of the question in regard to the date of publication, this combination is frequently incorrectly attributed to Zopf, Hueppe or even Schroeter.

⁴ This combination is attributed to Blanchard by Vuillemin (1913) without reference to the place of publication but search has thus far failed to show that he has ever used this combination.

⁵ Sometimes incorrectly ascribed to Bollinger.

⁶ Sometimes ascribed to Rivolta and Micellone (1879).

⁷ The combination *Actinocladothrix bovis* is frequently incorrectly ascribed to Afanasiev or to Afanasiev and Schultz.

- Nocardia actinomyces* Trevisan, 1889, p. 9.
Streptothrix actinomyces (Trevisan) Rossi-Doria, 1891, p. 405.
Cladothrix bovis (Harz) Macé, 1891, p. 666.⁸
Oospora bovis (Harz) Sauvageau and Radais, 1892, p. 271.
? *Actinomyces bovis-sulfureus* Gasperini, 1894, p. 684.
Nocardia bovis (Harz) Blanchard, 1896, p. 857.
Cladothrix actinomyces (Trevisan) Macé, 1897, p. 1038.
Streptothrix actinomyctica Foulerton, 1899, p. 780.
Streptothrix bovis communis Foulerton, 1901, p. 50.
Streptothrix bovis (Harz) Chester, 1901, p. 361.⁹
Sphaerotilus bovis (Harz) Engler, 1907, p. 5.

To this list must also be added, *Streptothrix isreali* Kruse, 1896, p. 56, *Actinomyces isreali* (Kruse) Lachnar-Sandoval 1898, p. 64, *Discomyces isreali* (Kruse) Gedoelst, 1902 p. 163 and *Cohnistreptothrix isreali* (Kruse) Pinoy, 1913, p. 931, in case investigations establish the fact that there is but one organism causing bovine actinomycosis.

Nocardia. This genus was first described in a paper (Trevisan, 1888) which is now very rare and apparently unobtainable in American libraries. The examination of a copy seen through the courtesy of Prof. C. Gorini of Milan shows that DeToni and Trevisan (1889) copied the portion of this paper describing this genus with little, if any, change. In the original paper, as in the paper by DeToni and Trevisan, five species are given in the genus, the first of these being *N. farcinica* Trevisan, the species described, but not named, by Nocard (1888). While this species is not definitely named as the type species, there is not the slightest question but that Trevisan regarded it as the type species of the new genus. *N. actinomyces* Trevisan (Syn. *Actinomyces bovis* Harz) is given as the second species followed by *N. foersteri* (Cohn) Trevisan (Syn. *Streptothrix foersteri* Cohn).

The species *N. farcinica* must therefore stand as the type species if the term *Nocardia* is used no matter what limits are set for the genus.

⁸ Sometimes incorrectly ascribed to Migula.

⁹ This combination is given as a synonym by Foulerton (1901) and it is possible that others used it before Chester.

Synonymy

Nocardia farcinica Trevisan, 1889, p. 9.¹⁰

Syn. *Streptothrix farcinica* (Trevisan) Rossi-Doria, 1891, p. 405.

Actinomyces farcinicus (Trevisan) Gasperini, 1892a, p. 222.

Oospora farcinica (Trevisan) Sauvageau and Radais, 1892, p. 248.

Actinomyces bovis farcinicus Gasperini, 1894, p. 684.

Cladothrix farcinica (Trevisan) Macé, 1897, p. 1047.

Streptothrix farcini bovis Kitt, 1899, p. 511.

Streptothrix nocardii Foulerton, 1901, p. 51.¹¹

Discomyces farcinicus (Trevisan) Gedoelst, 1902, p. 167.

Actinomyces nocardii (Foulerton) Buchanan, 1911, p. 378.

The combination *Bacillus farcinicus* apparently appears first in the literature as a synonym in a list given by Gasperini, 1892a, p. 183 where it is attributed to Nocard. Nocard appears, however, to have always used the expression "bacille du farcin," and never to have given a Latin name to this organism.

Cohnistreptothrix. Pinoy (1913) has named two species in this genus, *C. foersteri* and *C. isreali*, neither of which are specified as the type species of the genus. It is evident however from the form of the generic name that he wishes *C. foersteri* to be recognized as the type species and it should be so recognized by those who accept his subdivision of the genus.

SUMMARY

1. Because of confusion between *Streptothrix* Corda 1839 and *Streptothrix* Cohn 1875 and the general use of the former term by mycologists, the latter term should be generally disregarded. According to the International Rules of Botanical Nomenclature, the limited use of the term *Actinomyces* by Meyen in 1828 and 1832 is not sufficient to invalidate the generally used *Actinomyces* Harz 1877 provided the latter is accepted as a *genus conservandum* by an International Botanical Congress. The continued use of the latter term is therefore recommended. The type species of the genus is *A. bovis* Harz.

¹⁰ Frequently incorrectly attributed to DeToni and Trevisan.

¹¹ Incorrectly ascribed to Nocard by Foulerton.

2. The generic terms *Discomyces* Rivolta and *Actinocladothrix* Afanasiev and Schultz are and must remain synonyms of *Actinomyces* for all those who recognize the right of the International Botanical Congress to establish the validity of botanical names through legislative action. Those that contend that strict priority should govern the matter are apparently limited in their choice to the little used and confusing term *Discomyces*, a term repudiated even by its author. *Oospora* Wallroth, *Sphaerotilus* Kützing and *Cladothrix* Cohn do not properly apply to the organisms discussed in this paper.

3. There appears to be no justification for the use of the term *Nocardia* Trevisan for the entire group of organisms included in the Actinomycetaceae. It may however be properly used for a subdivision of the genus *Actinomyces*, provided however *N. farcinica* is retained in the genus *Nocardia* and is established as the type of the genus.

4. Knowledge of the group is however so imperfect that neither the subdivision of the group proposed by Wright (and accepted by the Committee on Classification and Characterization of the Society of American Bacteriologists) nor that proposed by Pinoy can be regarded as satisfactory.

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A CULTURE MEDIUM FOR THE MAINTENANCE OF STOCK CULTURES OF BACTERIA

M. COBB WORTH

Digestive Ferments Company, Detroit, Michigan

Received for publication, June 26, 1919

The magnitude of the question of the maintenance of stock cultures was brought to my attention by the work in the culture bureau at Parke, Davis and Company. Frequent transfers mean an immense amount of labor and material, for some cultures such as the meningococcus and gonococcus will live only about ten days even on special media such as ascitic agar. This means they must be transferred at least twice a week to be sure of keeping them viable and characteristic. *B. pertussis* and *B. influenzae* require weekly transfer and there are very few organisms that are safe to leave more than a month between transfers. It is evident, therefore, that the maintenance of cultures with the least amount of labor and material, so that all characteristic properties shall remain the same, is of interest and importance to those who are attempting to carry cultures in great number and variety.

Various media have been tried for special organisms, with considerable success, but a special medium for each different culture is a disadvantage in the smaller laboratories where media making is not carried on as a specialized art. In this work an attempt has been made to obtain some medium, as simple as possible, upon which various micro-organisms will remain viable and characteristic for a long time. While the work is by no means complete, it has been carried far enough and with enough success, to justify a report and a trial on a larger scale.

Drying has a tendency to attenuate or kill the majority of organisms, while on the other hand cultures in liquid media are rather difficult to handle and in many cases form disintegration

and by-products which ultimately become detrimental to the organisms themselves. Experiment has shown that mechanical protection is of value in the preserving of plant seeds and with this idea in mind, gelatin media of various acidities and contents were tested for their preserving qualities on organisms of various kinds.

Freshly inoculated gelatin cultures were incubated at 37°C., at which temperature the gelatin became liquid thus allowing the organisms to multiply and spread throughout the tube, forming a homogeneous mixture in twenty-four to forty-eight hours. Then these cultures were stored at room temperature, except the meningococcus and gonococcus which were kept at 37°C. The gelatin at room temperature partially solidified thus forming more or less of a mechanical protection for the organisms. Transfers were made periodically upon a suitable medium and special tests were made to see that the organisms had lost none of their characteristic properties, and as a control transfers were made from the same strains which had been kept by the regular method of frequent transfer upon suitable media.

The gonococcus and the meningococcus, *B. typhosus*, *B. paratyphosus* and *B. coli* were the organisms under most extensive and detailed study. *B. dysenteriae*, *B. diphtheriae*, *B. pseudodiphtheriae*, *B. pertussis*, *Micrococcus catarrhalis*, the Staphylococcus, and *Streptococcus viridans*, *S. fecalis* and *S. pyogenes* were also put under test.

The media under investigation were: I, regular gelatin, made according to standard methods, 1 per cent acid; II, regular gelatin plus 2 per cent glucose; III, regular gelatin 0.4 per cent acid; IV, regular gelatin 1 per cent acid plus 2 per cent glucose plus calcium carbonate; V, regular gelatin 1 per cent acid plus 2 per cent glucose plus rabbit liver.

A few months demonstrated that the more complex media had little or no advantage over the regular nutrient gelatin 1 per cent acid. The *B. typhosus*, *B. paratyphosus*, *B. coli*, *B. dysenteriae* and the meningococcus and gonococcus showed, if anything a preference for the 1 per cent acid gelatin. *B. pertussis* was not living at the end of one month on the regular gelatin plus 2 per

TABLE 1

Comparison of agglutinability of three strains of B. typhosus maintained for three months in gelatin and on plain agar

CULTURES	DILUTIONS OF ANTITYPHOID SERUM (RABBIT)							
	1-500	1-1000	1-2000	1-2500	1-3000	1-3500	1-4000	1-5000
No. 1 Sp..	+++	—	++	++	—	+	—	—
No. 1 R..	+++	—	+	+	—	+	—	—
No. 2 Sp..	+++	+++	+++	+++	+++	+++	+++	++
No. 2 R..	+++	+++	+++	++	++	++	++	+
No. 3 Sp..	+++	+++	+++	+++	+++	+++	+++	+++
No. 3 R..	+++	+++	+++	+++	+++	+++	+++	++

Sp = Cultures maintained in 1 per cent acid gelatin without transfer.

R = Cultures maintained in the regular manner, by transfers every three weeks on agar. *B. coli* was handled in the same way.

TABLE 2

Comparison of agglutinability of six strains of B. coli maintained for three months in gelatin and on plain agar

CULTURES	DILUTIONS OF WHITE SCOURS SERUM							
	1-500	1-1000	1-2000	1-4000	1-8000	1-16000	1-32000	CONTROL
No. 4 Sp..	+++	+++	+++	+++	+++	++	+	—
No. 4 R..	+++	+++	+++	+++	+++	++	+	—
No. 5 Sp..	+++	+++	+++	+++	++	+	—	—
No. 5 R..	+++	+++	+++	+++	++	+	—	—
No. 6 Sp..	—	—	—	—	—	—	—	—
No. 6 R..	—	—	—	—	—	—	—	—
No. 7 Sp..	+++	+++	+++	+++	++	+	—	—
No. 7 R..	+++	+++	+++	+++	+	—	—	—
No. 8 Sp..	+++	+++	+++	+++	+++	+++	++	—
No. 8 R..	+++	+++	+++	+++	+++	+++	++	—
No. 9 Sp..	++++	+++	+++	+++	+++	+++	++	—
No. 9 R..	+++	+++	+++	+++	+++	+++	++	—

cent glucose, while from the plain gelatins 1 per cent acid and 0.4 per cent acid good growths were obtained. Transfers from the eight months old cultures of *B. pertussis* in 1 per cent acid gelatin grew abundantly but with some involution forms; two months was about the limit of growth for *B. pertussis* on the 0.4 per cent acid gelatin.

Micrococcus catarrhalis gave an abundant growth upon transfer from both the 0.4 per cent acid gelatin and the 1 per cent acid gelatin plus 2 per cent glucose after three months.

Streptococcus viridans, at the end of one month, seemed in approximately the same condition on media I-II and III, while at the end of two months the cultures on the 0.4 per cent and 1 per cent acid gelatin seemed to have obtained the lead.

Hence most of the work from this point was carried along on 1 per cent acid gelatin.

At the end of three months, transfers were made onto plain agar slants from the gelatin cultures of *B. typhosus* nos. 2, and 3 and also from the corresponding cultures which had been maintained in the regular manner by transfer every three weeks on plain agar. The final transfer onto plain agar was not for rejuvenation but merely a means of getting the culture into a proper suspension for the agglutination test. At the end of twenty-four hours suspensions were made of these subcultures and the agglutination test gave the results shown in tables 1 and 2.

At the end of eight months *B. typhosus* nos. 2 and 3 were growing well, were microscopically characteristic and the agglutination tests were as shown in table 3.

TABLE 3

Comparison of agglutinability of two strains of B. typhosus maintained for eight months in gelatin and on plain agar

CULTURES	DILUTIONS OF ANTITYPHOID SERUM (RABBIT)						
	1-200	1-1000	1-1600	1-2000	1-3000	1-4000	Control
No. 2 Sp.	+++	++	+	—	—	—	—
No. 2 R.	+++	+++	+	—	—	—	—
No. 3 Sp.	+++	+++	+	—	—	—	—
No. 3 R.	++++	+++	+	—	—	—	—

At the end of eleven months the *B. coli* were growing well and were microscopically characteristic. The agglutination tests were as shown in table 4.

TABLE 4

Comparison of agglutinability of three strains of B. coli maintained for eleven months in gelatin and on plain agar

CULTURES	DILUTIONS OF WHITE SCOURS SERUM						
	1-200	1-2000	1-10,000	1-20,000	1-24,000	1-30,000	Control
No. 4 Sp.	+++++	++++	+	—	—	—	—
No. 4 R.	+++++	++++	+?	+	+	—	—
No. 8 Sp.	+++++	++++	++++	++	++	—	—
No. 8 R.	+++++	++++	++++	++	++	—	—
No. 9 Sp.	+++++	+++++	++++	+++	++?	—	—
No. 9 R.	+++++	+++++	++++	++++?	++?	—	—

The meningococcus and gonococcus were planted on 1 per cent acid gelatin, stored at 37°C. and tested at the end of three months for viability, microscopical appearance and serum reaction. All cultures were found in first class condition. At the end of seven months these cultures were again tested, good growths were obtained, of characteristic microscopical appearance, and the serum reactions were as shown in table 5.

TABLE 5

Comparison of agglutinability of one strain of meningococcus maintained for seven months in gelatin and on ascitic agar

CULTURES	DILUTIONS OF ANTIMENINGOCOCCIC SERUM						
	1-20	1-50	1-100	1-200	1-400	1-600	Control
No. 10 Sp.	+++	+++	+++	++	+	?	—
No. 10 R.	+++	+++	+++	++	+	?	—

TABLE 6

Comparison of agglutinability of four strains of meningococcus maintained for eight months in gelatin and on ascitic agar

CULTURES	DILUTIONS OF ANTIMENINGOCOCCIC SERUM						
	1-50	1-200	1-400	1-600	1-800	1-1000	Control
No. 11 Sp.	+++++	+++++	+++	+++	++?	—	—
No. 11 R.	+++++	+++++	+++	+++	++?	—	—
No. 12 Sp.	+++++	+++++	+++	+++	+++	+++	—
No. 12 R.	+++++	+++++	+++	+++	+++	+	—
No. 13 Sp.	+++++	+++++	+++++	+++++	+++++	+++	—
No. 13 R.	+++++	+++++	+++++	+++++	+++++	+++	—
No. 14 Sp.	+++++	+++++	+++	+++	++	+	—
No. 14 R.	+++++	+++++	+++	+++	++	+	—

At the end of eight months the meningococci were again tested and found growing well; they showed characteristic microscopical appearance and the serum reactions were as shown in table 6.

SUMMARY

A medium of the following composition has been found favorable for the preservation of *B. typhosus*, *B. paratyphosus*, *B. coli*, *B. dysenteriae*, *B. pertussis*, *Micrococcus catarrhalis*, a streptococcus and the meningococcus and gonococcus for several months without transfer:

Nutrient Gelatin:	
Chopped beef.....	500 grams
Water.....	1000 cc.
Heated in a water bath 50-55°C. for 1 hour.	
Strained through bag cloth, volume restored:	
Peptone.....	10 grams
NaCl.....	5 grams
Gelatin.....	100 grams
Dissolved, filtered, adjusted to 1 per cent acid.*	
Sterilized for twenty minutes at 110°C.	

Inoculation is made and the cultures are placed at 37°C. for twenty-four hours after which they are stored at 20°C. with the exception of the meningococcus and gonococcus which are kept at 37°C.

Without further attention *B. typhosus* retained its viability, characteristic appearance, and agglutinating power for eight months; *B. coli* for eleven months; meningococcus for eight months. The serum reactions were not determined on cultures except as reported. *B. paratyphosus*, *B. pertussis* and *B. dysenteriae* retained their viability and characteristic appearance for eight months. The streptococcus was still in good condition at the end of four months.

* In the continuation of this work in the laboratories of the Digestive Ferments Company, reactions are measured in terms of H-ion concentrations, which standard of measurement will be used in the future.

THE VALUE OF PRESUMPTIVE TESTS FOR *BACILLUS COLI* BASED ON THE ROUTINE USE OF LACTOSE BILE AND LACTOSE BROTH

NEVA RITTER

From the Department of Bacteriology, University of Kansas, and the Water and Sewage Laboratory, Kansas State Board of Health

Received for publication, July 5, 1919

Previous to 1906, glucose broth was generally used in the presumptive test for *Bacillus coli*. As a result of the work of Jackson (1906, 1907), Weston and Tarbett (1907), Sawin (1907), and others (Hale and Melia, 1910), lactose peptone bile was substituted for glucose broth by the American Public Health Association in the 1912 Standard Methods of Water Analysis. Doubt was, however, thrown upon the accuracy of the results obtained by the use of lactose bile by Jordan (1913) and Obst (1916), which resulted in another change by the American Public Health Association in 1917, lactose broth being substituted for lactose bile. The authors cited showed that lactose bile not only inhibited organisms outside of the typhoid-colon group, but that it also had an inhibitive action on members of this group itself.

In this laboratory, both bile and broth are used for the detection of *B. coli* in tap waters; and the present study is based on examinations of 1899 samples of tap water showing fermentation in bile, or broth, or both, from the forty-six city surface supplies in Kansas, of which weekly tests are made. These cover the period from July 1, 1918, to May 15, 1919.

The bile used is prepared by diluting oxgall 50 per cent with distilled water and adding 1 per cent lactose and 1 per cent peptone. Both large and small culture tubes with inverted vial fermentation tubes are filled,— the large tubes with 15 cc. of medium and the small ones with 7 cc.

The broth, which is tubed in the large fermentation tubes, contains 0.3 per cent of Liebig's Beef Extract, 1 per cent peptone and 1 per cent lactose, and is adjusted to a point neutral to phenolphthalein.

Endo plates are made of 3 per cent agar with 1 per cent lactose and 0.37 per cent fuchsin decolorized with 5 per cent sodium sulphite. The reaction is adjusted to 0.5 per cent acid to phenolphthalein. This amount of fuchsin gives a rapid and brilliant coloration with acid formers, and a good metallic sheen on the colonies of *B. coli*.

Each sample is inoculated as follows: 1 cc. into each of five small bile tubes, 10 cc. into one large bile tube, 10 cc. into each of three large broth tubes.

These are incubated at 37° and after twenty-four hours, the cultures are read; those showing 10 per cent or more gas in the fermentation tube, or showing active fermentation, being streaked on Endo plates for confirmation, and the remaining cultures being returned to the incubator until forty-eight hours have elapsed. They are then read once more, positive cultures confirmed, and all discarded. Endo plates are incubated about twenty hours.

In recording results, twenty-four hour readings are marked in red ink, forty-eight hour readings in blue ink, with + and - to show presence or absence of gas. To indicate a 10 cc. sample, a circle is placed around the symbol. Bile results are always recorded above broth to avoid confusion.

Table 1 shows the relationship between confirmatory tests and the presence of gas in one or both of the bile and broth media. These results have been studied in an effort to learn if the simultaneous use of the two media might make confirmation unnecessary.

When both media are used and both show fermentation, 75.05 per cent confirm in either bile, broth, or both media. When bile alone shows fermentation, which is in a very small number of samples, 34.42 per cent confirm, as opposed to 25.20 per cent when broth alone is positive, which occurs in ten times as many cases. According to these results when both media are used in the presumptive test, there are fewer mistakes than when either is used separately.

TABLE 1

	NUMBER SAMPLES BILE AND BROTH FERMENT- ING	NUMBER SAMPLES BILE AND BROTH CONFIRM- ING	PER CENT SAMPLES BILE AND BROTH CONFIRM- ING	NUMBER SAMPLES BILE ONLY CONFIRM- ING	PER CENT SAMPLES BILE ONLY CONFIRM- ING	NUMBER SAMPLES BROTH ONLY CONFIRM- ING	PER CENT SAMPLES BROTH ONLY CONFIRM- ING	TOTAL PER CENT CONFIRM- ING
Bile, +... Broth, +.	1223	752	61.48	68	5.56	98	8.01	75.05
Bile, +... Broth, -	61			21	34.43			34.42
Bile, -... Broth, +.	615					155	25.20	25.20

The next step was to examine the results to determine if the number of broth tubes showing fermentation is any indication of the presence of typical *B. coli*.

It will be noted from table 2 that when all three broth tubes are positive, the percentage confirmed is above that calculated

TABLE 2

	NUMBER SAMPLES BILE AND BROTH FERMENT- ING	NUMBER SAMPLES BILE AND BROTH CONFIRM- ING	PER CENT SAMPLES BILE AND BROTH CONFIRM- ING	NUMBER SAMPLES BILE ONLY CONFIRM- ING	PER CENT SAMPLES BILE ONLY CONFIRM- ING	NUMBER SAMPLES BROTH ONLY CONFIRM- ING	PER CENT SAMPLES BROTH ONLY CONFIRM- ING	TOTAL PER CENT CONFIRM- ING
Bile, +... Broth, 3+	888	618	69.59	37	4.16	66	7.43	81.18
Bile, +... Broth, 2+	193	79	40.93	15	7.77	23	11.91	60.61
Bile, +... Broth, 1+	131	49	37.40	13	9.92	10	7.63	54.95
Bile, -... Broth, 3+	178					52	29.26	29.26
Bile, -... Broth, 2+	158					36	22.78	22.78
Bile, -... Broth, 1+	289					65	22.49	22.49

for the whole number of tests with both media fermenting, the average in the former being 81.18 per cent and in the latter 75.05 per cent. When only two of the three tubes are positive, 60.61 per cent confirm, and when only one ferments 54.95 per cent confirm. If both media are used and there is fermentation in any of the bile and all of the broth tubes, fewer unconfirmed presumptive tests will be obtained than in the cases where the number of tubes reacting positively is one or two.

TABLE 3

	NUMBER SAMPLES BILE AND BROTH FERMENTING	NUMBER SAMPLES BILE AND BROTH CONFIRMING	PER CENT SAMPLES BILE AND BROTH CONFIRMING	NUMBER SAMPLES BILE ONLY CON- FIRMING	PER CENT SAMPLES BILE ONLY CON- FIRMING	NUMBER SAMPLES BROTH ONLY CONFIRMING	PER CENT SAMPLES BROTH ONLY CONFIRMING	TOTAL PER CENT CONFIRMING
Bile, +.								
Broth, +, 24 hours.	387	362	93 54	6	1.55	10	2.58	97.67
Bile, +, 24 hours...	8			3	37.50			37.50
Broth, -								
Bile, -								
Broth, +, 24 hours.	42					23	54.76	54.76
Bile, +, 24 hours...								
Broth, +, 48 hours	82	50	60.75	7	8.53	4	4.89	83.17
Bile, +, 48 hours..								
Broth, +, 24 hours	124	86	69 35	3	2.43	24	19 35	91.13

Another point for consideration is whether or not this same conclusion applies when the broth only is positive. By comparing the last three percentages in the preceding table this is seen to be the case, as when three tubes are positive there is 29.26 per cent confirmation, when two are positive, 22.78 per cent when only one is positive, 22.49 per cent, respectively.

A study of the time factor was next made and summarized in table 3.

There is no need for confirmation when both bile and broth show fermentation in twenty-four hours, as 97.67 per cent of

the samples so reacting proved to be positive in either bile, broth, or both media. If either bile or broth is positive in twenty-four hours and the other in forty-eight hours, there is a higher percentage of samples containing *B. coli* than when both bile and broth are positive only after forty-eight hours, as is shown by 83.17 per cent of confirmation with bile positive in twenty-four hours and broth in forty-eight hours, and 91.13 per cent with broth positive in twenty-four hours and bile in forty-eight hours.

This would indicate that while bile is fermented more slowly than broth, yet in dealing with contaminated waters it is just as accurate.

The statistics of these results on 1899 samples of surface waters examined for pollution, where both bile and broth are used simultaneously, would suggest the following conclusions:

1. If there is fermentation in both bile and broth tubes, the presumptive test is reliable in 75.05 per cent of all cases considered

2. If all three broth tubes, as well as the bile, are positive, a greater percentage of tests are confirmed as *B. coli* than is the case when only one or two tubes out of three are positive.

3. When broth alone is positive the water in 70 per cent to 78 per cent of cases is proved not to contain *B. coli*.

4. Samples with both media positive in twenty-four hours contain *B. coli* in 97.67 per cent of cases and therefore do not need confirmation.

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MORPHOLOGICAL CHANGES DURING THE GROWTH OF BACTERIA

PAUL F. CLARK AND W. H. RUEHL

From the Bacteriological Laboratory, Medical School, University of Wisconsin, Madison

Received for publication, July 5, 1919

The life cycle of bacteria has been studied by a number of different methods. Some investigators (Müller, 1895, Lane-Clayton, 1909, Chick, 1912, Chesney, 1916, etc.) have determined the numbers of viable organisms which may be present at different periods following the transplantation of the organisms to fluid culture media. In such examinations, certain periods or phases of growth have been observed. Lane-Clayton recognized four phases in the life of a bacterial culture: (1) the latent period, or the initial phase of slow growth or no growth at all; (2) the period of maximum rate of growth, or logarithmic period, when the organisms are dividing regularly; (3) the stationary period in which the number of organisms remains more or less constant; (4) the period of decline when the numbers of living bacteria are diminishing. Buchanan (1918), analyzing the situation somewhat more closely, designates seven growth phases:

1. Initial stationary phase.
2. Positive growth acceleration phase or period of lag during which the average rate of increase in numbers of organisms increases with the time.
3. Logarithmic growth phase. During this phase the minimal average generation time and therefore the maximum rate of division is maintained throughout the period.
4. Phase of negative growth acceleration, during which the rate of division is decreased.

5. Maximum stationary phase during which there is practically no increase in the number of bacteria.

6. Phase of accelerated death. During this phase the numbers of viable bacteria are decreasing.

7. Logarithmic death phase in which the rate of death remains constant.

These phases of growth of a bacterial culture, however they may be designated, obviously merge into each other, the duration of each phase varying somewhat depending upon the organism and the different growth conditions.

Other observers have attempted to obtain information in regard to possible growth cycles among bacteria by studying the development of individual bacteria in hanging block and hanging drop cultures. Some interesting details of the simple cell division of bacteria have been observed, such as the demonstration of the division of the nuclear substance preceding that of the cell body by Nakanishi (1901) and the peculiar division displayed by *B. diphtheriae* in which the split occurs at a point occupied by a metachromatic granule (Williams, 1917).

But with all the observations on the development of bacteria and bacterial cultures, not until recently has anyone found evidence of the existence of complicated life cycles similar to those found among higher Thallophytes or among the Protozoa. Löhnis and Smith (1916) as the result of painstaking researches have, however, reported the finding of such complex cycles in many species of bacteria. They state that all bacteria studied live alternately in an organized and in an amorphous or "symplastic" stage; that bacteria multiply not only by fission, but also by the formation of filterable gónidia, which subsequently germinate either directly or after having entered the symplastic stage. In the symplastic stage they assume the probability of conjugation and describe many complex developmental stages.

The reports of Rosenow (1914) affirming the transmutation of streptococci into pneumococci, and the reverse transformation, and the possibility of transforming a typical diphtheroid organism into a minute coccoid organism and back again (Bunting and Neumann, personal communication) also lend additional

interest to the question of the fixity both of the delicate biologic adjustments of bacteria and of their morphology. That the question is more than an academic one is obvious. Can the bovine tubercle bacillus become metamorphosed into the human type? Can the streptococcus from a streptococcus throat become changed so as to produce a typical scarlet fever, or is the typical scarlet fever merely a different reaction on the part of the individual infected? etc.

It seemed to the authors that some additional information in regard to possible cyclical changes in the development of bacteria might be obtained by making repeated microscopic examinations of organisms at intervals of one to three hours for twenty-four hours after transplanting, and at longer intervals for a week or more.

In all the experiments careful attention was paid to the purity of the original cultures, repeated platings being used in doubtful cases. Typical organisms were transplanted daily to accustom them to the media used and also to eliminate as far as possible the period of "lag" (Barber (1908)). In each series the organisms were grown on tubes from the same lot of media. When in an excellent vegetative state transplants were made. In the first large group of cases a twenty-four hour culture was used as the parent culture, and, in the second group, a forty-eight hour culture. To this parent culture 2 cc. of broth were added and with the resulting suspension four or more tubes were inoculated by seeding with a pipette 0.1 cc. to each tube. The media and broth used were taken directly from the refrigerator and all tubes were placed in the incubator at 37.5°C. at the same time.

In the first group of observations smears were made from the original parent culture and from transplants at the end of 1, 2, 4, 6, 9, 12, 15, 18, 21, 24 and 48 hours and on the seventh day. In each case during the first twelve hours the smear was made from a different and previously untouched culture. The smears made at the fifteen and eighteen hour periods were made from the same tube, care being observed to take the organisms from a different portion of the tube in each case. The rest of the smears were made from cultures that had been used during the

early hours of growth, as it was felt that this disturbance would no longer be an appreciable factor. To insure uniformity of staining, the smears of each strain were for the most part made on a single slide and stained in one operation. Methylene blue was generally used. Observations were made as to size, shape, characteristic grouping, and variation in staining of the bacteria during the development of the cultures.

During these observations, made on a number of strains of many different species of bacteria, it became obvious that the most interesting changes occurred during the first twelve hours of growth. In subsequent observations, therefore, the eighteen and twenty-one hour smears were omitted; thus the necessity of night work was avoided. In addition to the more general observations, careful measurements were made of the organisms from the cultures of different ages by the usual stage and ocular micrometer method. Especial care was taken not to consider two attached organisms as one individual. To avoid error due to individual variations in the organisms, ten organisms selected at random from several different fields were measured. The average of the ten was taken and this average length was used in the construction of curves representing the size of the organisms throughout the growth period. Notation was also made of the smallest and largest organisms observed in the series of ten organisms measured.

Seventy strains were used in all, comprising thirty-seven species. These organisms were obtained either from the American Museum of Natural History, our own collection of stock cultures, or from the other sources mentioned in the appended list.

Coccaceae

1. *Albococcus candicans* no. 260 (American Museum)
2. *Albococcus candicans* no. 174 (American Museum)
3. *Aurococcus mollis* no. 207 (American Museum)
4. *Aurococcus mollis* no. 4 (American Museum)
5. *Staphylococcus aureus* no. 347 (American Museum)
6. *Staphylococcus aureus* (recently isolated stock)
7. *Staphylococcus aureus* (laboratory stock)
8. *Streptococcus M. S.* (Dr. Rosenow)

9. *Streptococcus* no. 736 (Dr. Rosenow)
10. *Streptococcus pyogenes* no. 1 (laboratory stock)
11. *Streptococcus pyogenes* no. 2 (laboratory stock)
12. *Streptococcus pyogenes* no. 3 (laboratory stock)
13. *Streptococcus pyogenes* no. 4 (laboratory stock)
14. *Diplococcus pneumoniae* Type I (Rockefeller Hospital)
15. *Diplococcus pneumoniae* Type II (Rockefeller Hospital)
16. *Diplococcus pneumoniae* Type III (Rockefeller Hospital)
17. *Diplococcus mucosus* no. 92 (American Museum)
18. *Diplococcus pneumoniae* no. 93 (American Museum)
19. *Diplococcus pneumoniae* (from milk)
20. *Diplococcus gonorrhoeae* no. 288 (American Museum)
21. *Diplococcus catarrhalis* no. 649 (American Museum)
22. A Gram negative coccus (not identified)
23. *Diplococcus catarrhalis* (laboratory stock)
24. *Diplococcus weichselbaumii* (laboratory stock)

Bacteriaceae

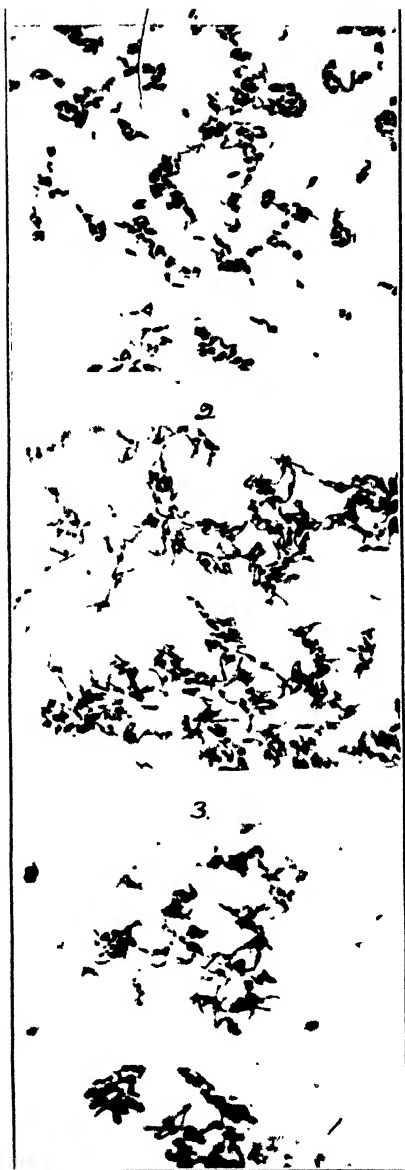
25. *B. coli-communis* (laboratory stock)
26. *B. coli-communior* (laboratory stock)
27. *B. typhosus* no. 0 (Wisconsin State Laboratory of Hygiene)
28. *B. typhosus* no. 3 (Johns Hopkins)
29. *B. paratyphosus* A (Wisconsin State Laboratory of Hygiene)
30. *B. paratyphosus* B (Wisconsin State Laboratory of Hygiene)
31. *B. typhosus* no. 1 (laboratory stock)
32. *B. typhosus* no. 2 (laboratory stock)
33. *B. typhosus* no. 4 (laboratory stock)
34. *B. dysenteriae* (laboratory stock)
35. *B. paradysenteriae* (laboratory stock)
36. *B. suipestifer* (laboratory stock)
37. *B. of Friedlander* (laboratory stock)
38. *B. capsulatus* (laboratory stock)
39. *B. proteus* (laboratory stock)
40. *B. prodigiosus* (laboratory stock)
41. *B. pyocyaneus* (laboratory stock)
42. *B. avisepticus* (American Museum)
43. *B. mallei* (American Museum)
44. *B. diphtheriae* no. 1 (laboratory stock)
45. *B. diphtheriae* no. c (laboratory stock)
46. *B. diphtheriae* no. Sp (laboratory stock)
47. *B. diphtheriae* Park no. 8 (American Museum)
48. *B. xerosis*
49. *B. hofmanni* (laboratory stock)
50. *B. hodgkini* (Corbett) (Dr. Bunting)
51. *B. hodgkini* E₃ (Dr. Bunting)
52. *B. hodgkini* (Blessing) (Dr. Bunting)
53. *B. hodgkini* B₃ (Dr. Bunting)

- 54. *B. hodgkini* M (Dr. Bunting)
- 55. *B. hodgkini* Endo (Dr. Bunting)
- 56. *B. hodgkini* Banti II (Dr. Bunting)
- 57. *B. anthracis* (American Museum)
- 58. *B. anthracoides* (American Museum)
- 59. *B. megatherium* (American Museum)
- 60. *B. mycoides* (American Museum)
- 61. *B. subtilis* (American Museum)
- 62. *B. vulgatus* (American Museum)
- 63. *B. leprae* (American Museum)
- 64. *B. smegmatis* (American Museum)
- 65. *Grass bacillus* (American Museum)
- 66. *B. pertussis* (American Museum)
- 67. *B. influenzae* (American Museum)

Spirillaceae

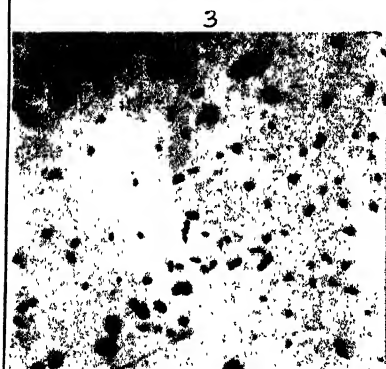
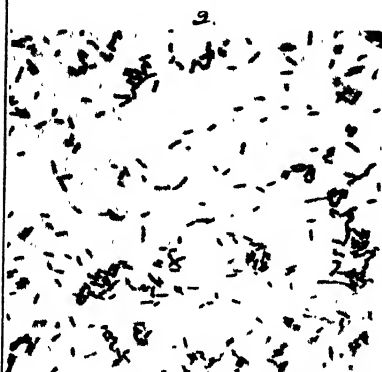
- 68. *Sp. metchnikovi* (American Museum)
- 69. *Sp. cholerae* (American Museum)
- 70. *Sp. schuykilliensis* (American Museum)

As a result of these examinations we observed, in all the groups of bacteria studied, striking changes in morphology, especially during the early hours (two to six) of the development of the cultures. In some of the strains no apparent change could be seen for the first two hours, but in the majority of instances even at the end of this initial period a definite increase in size was observed, the organisms took the stain more intensely, and their outline was more sharply defined. Following this, the growth in size was rapid. Many chains of organisms were observed in cultures where they are usually absent. A majority of the organisms from a culture of *Bacillus prodigiosus* four hours old are as large and coarse as the common vegetative forms of the hay bacillus. They will average 1.3 micra in length and 0.7 to 0.8 micra in width. Many of the young, vigorous, rapidly dividing typhoid bacilli from a six hour culture are 4 to 6 micra long and 0.7 to 0.8 micra wide, so thick and clumsy that only further examinations convince an observer that he is dealing with a pure culture of *B. typhosus*. Again, cultures of *Staphylococcus aureus* at this period (four to six hours) present very large, heavy cocci, many of them twice the diameter of the cocci more usually seen. The usual time when the average size



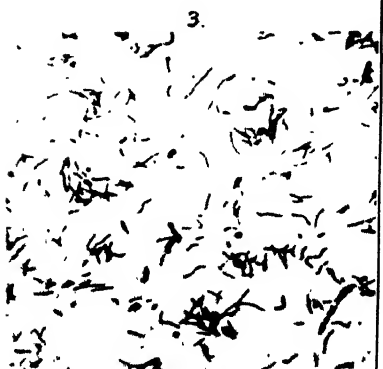
B. DIPHTHERIAE (PARK No 8). $\times 800$

1. Smear from 4 hour culture
2. Smear from 21 hour culture
3. Smear from 48 hour culture



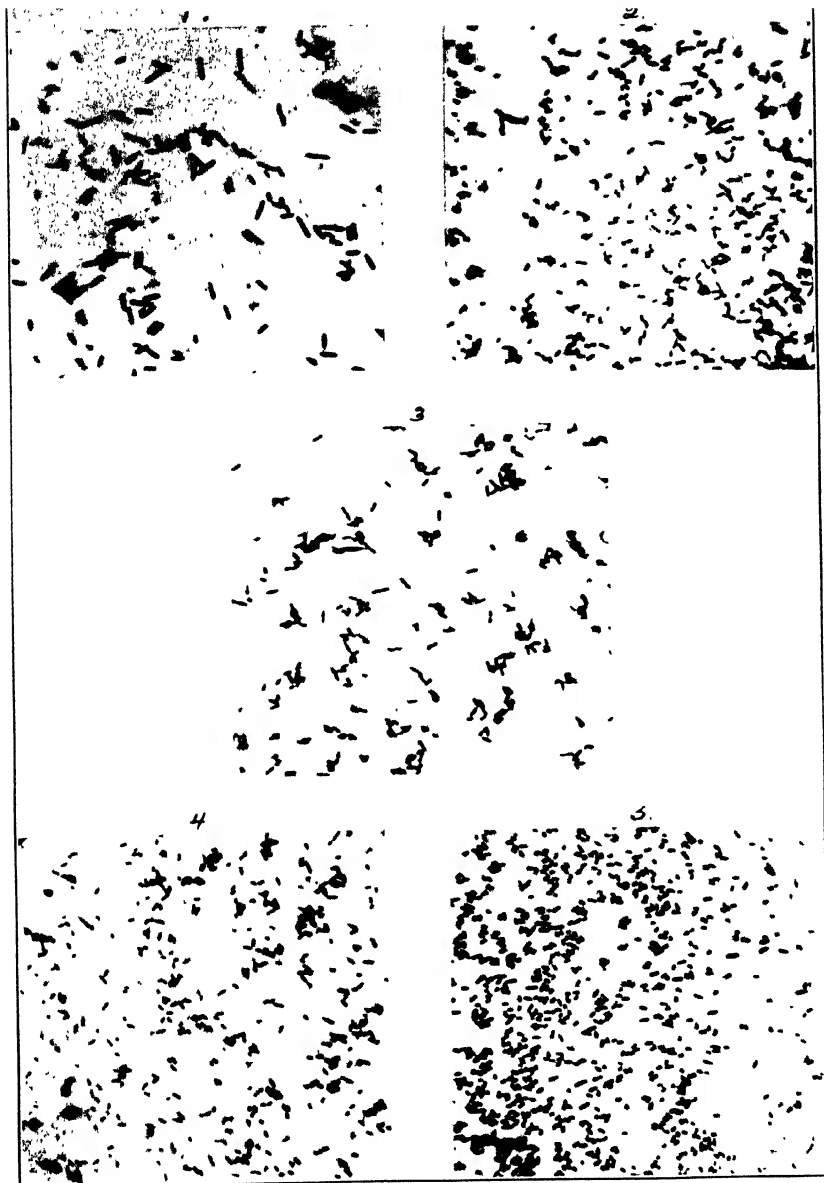
B. PRODIGIOSUS. $\times 800$

1. Smear from 2 hour culture
2. Smear from 4 hour culture
3. Smear from 12 hour culture



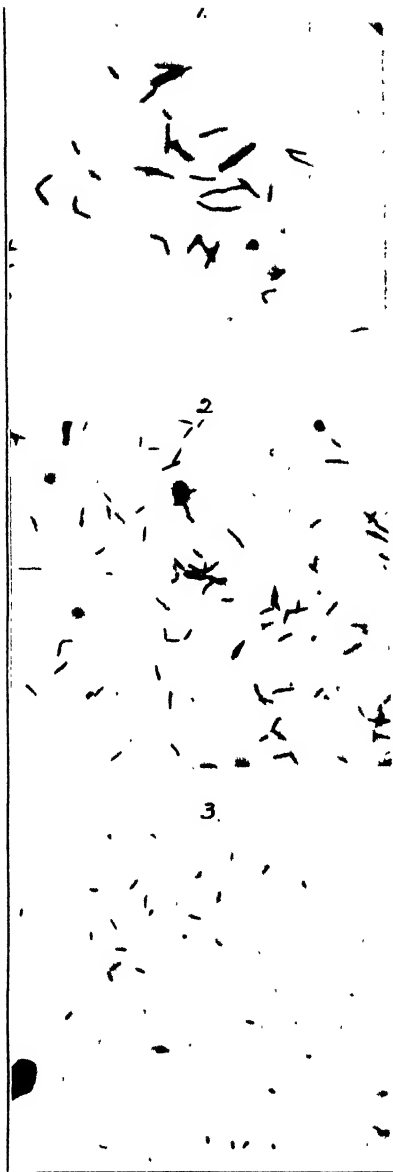
SP. CHOLERAЕ. $\times 800$

1. Smear from 2 hour culture
2. Smear from 12 hour culture
3. Smear from 24 hour culture



B. TYPHOSUS No. 0. $\times 800$

- | | |
|-------------------------------|-------------------------------|
| 1. Smear from 4 hour culture | 3. Smear from 15 hour culture |
| 2. Smear from 9 hour culture | 4. Smear from 21 hour culture |
| 5. Smear from 48 hour culture | |



B. LEPRAE. $\times 800$

1. Smear from 6 hour culture
2. Smear from 12 hour culture
3. Smear from 168 hour culture



B. SUBTILIS. $\times 800$

1. Smear from 6 hour culture
2. Smear from 9 hour culture
3. Smear from 18 hour culture

of the organisms reached its maximum was four to six hours after transplantation. In some instances, however (a strain of *B. coli-communior* for example), the height of the curve was reached in the two hour cultures, and in others (Hopkins strain of *B. typhosus*) not until the cultures had been growing for nine hours. Throughout this period when the organisms are passing, presumably, through the second and third phases according to Buchanan, (1918) the microscopic picture is irregular. The minimum average generation time is short so that the cross section of this rapidly growing culture, which is of course what we obtain when we take a smear, presents organisms in all stages of development, but a majority of them are large and ready for further division.

As the cultures grow older the microscopic picture changes. Smears from the nine or twelve hour cultures present organisms of a smaller average size, this average dwindling, until by the time the cultures are eighteen to twenty-four hours old the classical text-book picture is presented. During this period growth and consequent reproduction are apparently taking place much less rapidly. Chains of bacilli, so prevalent in the younger cultures of the non-chain forming organisms, were not observed. Irregular forms were few in number and a marked uniformity in the size of the organisms was evident. Characteristic grouping manifested itself and the staining was even.

Cultures older than twenty-four and forty-eight hours presented more and more of the involution forms described by many authors. Irregular staining, in many instances bizarre forms, and organisms averaging smaller than those found in the twenty-four hour cultures predominated.

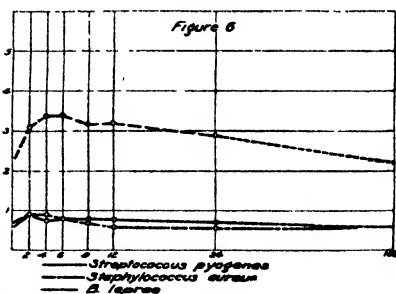
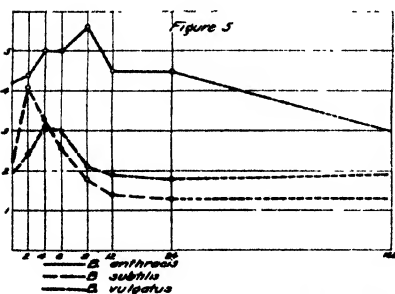
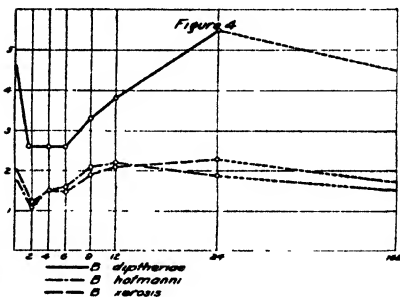
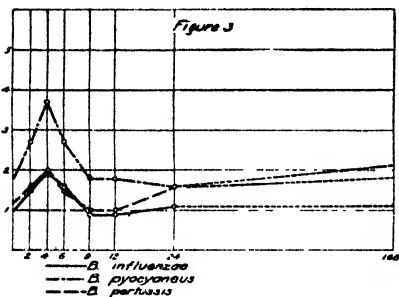
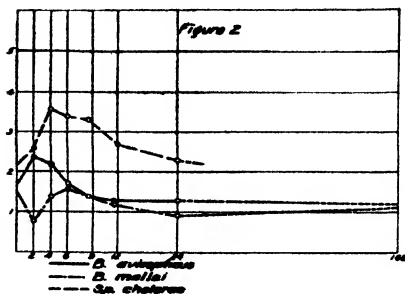
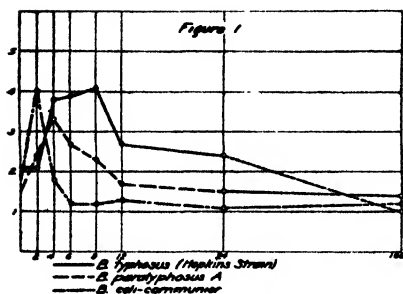
Members of the diphtheria group were the only bacteria (with the possible exception of *B. mallei*) among those examined that did not conform to the general order of morphological changes noted above. In this group, however, the majority of the organisms in the young cultures (two to six hours) were definitely smaller than the organisms transplanted. In fact, in most strains the original average size was not regained until twelve to eighteen hours had elapsed. Not only were the major-

ity of the organisms in the young cultures smaller than those transplanted, but they were also definitely smaller than the involution forms found in cultures six or seven days old. In no other group of organisms did this obtain. In all the others, the old "senile" forms so commonly present in old cultures showed the smallest average size. The peculiar pleomorphism, irregular staining, metachromatic bars and granules so characteristic of the diphtheria group did not ordinarily appear to any great extent until the cultures were fifteen to twenty-one hours old.

The graphs presented (figs. 1-6) are representative of the growth curves obtained and are self-explanatory. The photomicrographs also show clearly the striking differences in the morphology of the different organisms from cultures of different ages.

There would seem to be little need for prolonged discussion of these simple observations. The correlation in time of the occurrence of the long coarse forms with the period when the maximum growth of the culture is taking place, is obvious. That the cross section of the culture with its very short average generation time should show a majority of forms large and nearly ready for further divisions is also obvious. Just why the diphtheria group should depart from the general rule as to the size of the organisms in young cultures is not clear.

The more fundamental question, the reason for the cyclical growth of a bacterial culture or a culture of *Paramoecium* or other living organisms, is not touched upon by the present observations. As far as the bacterial culture is concerned the conception that the exhaustion of the food supply is an important factor, was overthrown very early. A more adequate conception, generally accepted for many years, is that the bacteria form toxic substances which later inhibit the growth of the organisms themselves. Chesney (1916) has offered strong evidence that lag is also an expression of this injury which the bacterial cell has received in its previous environment. It has long been recognized that many toxic substances which in large concentrations will cause necrosis of the cell will in small concentrations cause proliferation of that cell. Indeed in many,




ORDINATES - Length of Bacteria in Microns
 ABSCISSAS - Time in Hours

possibly all, pathological processes which result in a simple hypertrophy or in hyperplasia, it is recognized that injury to the mother cells is the necessary stimulus which initiates this reaction. Jennings has shown that even fertilization in the Infusoria is a stimulus partaking largely of the nature of an injury. Furthermore, Loeb has shown that by mechanical injury to the unfertilized frog's egg he could stimulate cell division and produce an artificial parthenogenetic frog. May it not be possible that the toxic substance which in large amounts cause the death of the bacterial culture and in smaller amounts produce the phenomenon of lag, act when diluted and diffused in a fresh tube of culture medium as the slightly injurious stimulus for the rapid cell division which takes place?

CONCLUSIONS

1. The morphology of seventy strains of organisms, representing thirty-seven species selected from most of the recognized groups of pathogenic bacteria, has been studied at brief intervals for a period of forty-eight hours and at longer intervals for a week.

2. In all instances save among the members of the diphtheria group, and possibly *B. mallei*, the young organisms found in cultures from four to nine hours old are much larger than the forms found in the cultures twenty to twenty-four hours old. In some instances the difference is so great as to render the organisms unrecognizable when viewed by the ordinary standard of the twenty-four hour culture.

3. The period when the largest organisms are found corresponds closely to the period when rapid cell division is taking place as shown by the numbers of viable organisms. 

4. In the diphtheria group, however, the young organisms in cultures from four to nine hours old are definitely smaller and more solid staining than the older forms. In fact, the average size in cultures of this age is distinctly less than in the case of very old cultures showing involution forms. In the other groups the senile forms are always the smallest.

5. It is important to recognize the morphological changes in the familiar pathogenic bacteria and to note that these changes occur correlatively with the rapidity of increase in the numbers of organisms.

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